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Manganese (Mn) is a trace metal that is essential for optimal functioning of mammalian systems. However, excessive exposure to Mn is known to cause an extrapyramidal disease. Presently, Mn-induced neurotoxicity has no cure. In these studies, we examined the effectiveness of creatine therapy on models of Mn neurotoxicity. Primary astrocytes were cultured and divided into five groups: controls (CN), Mn group (300 ε M $MnCl_2$ for 24 hour), creatine group (1 mM creatine monohydrate for 24 hour), Mn followed by creatine treatment group (MnCr), and creatine treatment followed by Mn exposure group (CrMn). Results showed a significant increase in Mn concentrations in Mn and CrMn groups ($p < 0.05$), with Mn group showing a significant decrease in cellular viability and creatine attenuating the toxic effects of Mn in the MnCr and CrMn groups. A significant elevation in glutamate-aspartate transporter gene expression was seen in CrMn compared to CN, and glutathione peroxidase (GPx) gene expression was significantly decreased in MnCr and CrMn groups. Based on this study, we concluded that creatine may have some sort of neuroprotection in Mn-exposed primary cultured astrocytes. Following the astrocyte study, four brain regions from male Sprague-Dawley rats exposed to 1 g $MnCl_2$ /L in water (Mn; n=6), given intraperitoneal injections of 75 g/kg body weight monohydrate creatine (Cr; n=6), or exposed to Mn and creatine injections (MnCr; n=6), or received no creatine injection with no exposure to Mn (CN; n=6) were dissected and processed. The effect of waterborne exposure to Mn and creatine treatment on gene expression profiles showed a statistical trend for an increase in

glutamate-transporter-1 gene expression in MnCr group in the globus pallidus (GP) (p=0.066) and caudate-putamen (CP) (p=0.052) when compared to CN. A statistical trend for increased GPx gene expression was observed in Cr group of the substania nigra (SN) (p= 0.055) and MnCr group of cortex (CX) (p=0.051). Heme oxygenase-1 (HMX-1) in the Mn group of SN showed a statistical trend for increased gene expression (p= 0.072) when compared to CN, and GP showed a statistical trend towards an increased HMX-1 expression in Mn group (p = 0.08) when compared to MnCr group. Finally, brain cytosolic creatine kinase gene expression was significantly lowered in the MnCr group of SN when compared to CN. Our study suggests that subchronic waterborne exposure to Mn does not cause significant changes on markers of oxidative stress and creatine treatment exerts some neuroprotection overall.

CREATINE AS A NEUROPROTECTIVE AGENT IN MANGANESE-INDUCED NEUROTOXICITY

By

Shatha I. Farah

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Approved by

_____
Committee Chair

DEDICATION

To those who contribute to my life story; the greatest gifts that one could ever receive.

To my best friend and fiancé, Fadi Jimblat.

To my family and friends.

To Dr. Keith Erikson and future Dr. Steve Fordahl

To the Nutrition Department at UNCG.

This project is dedicated for all of you who have supported me day by day, and directed me step by step until I reached my goal and achieved my dream of obtaining this degree.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Manganese (Mn) is an essential metal that is required in low concentrations for normal physiological functioning. However, excessive exposure to Mn through air or water results in its toxic accumulation in the brain leading to a parkinsonian syndrome known as manganism. The risk of exposure to toxic levels of Mn is not only occupational; but rather it has become an environmental concern, and the risk includes patients with liver disease, iron-deficient individuals, and even children. In adults, behavioral changes are early manifestations of Mn neurotoxicity, which later progress into neurological motor abnormalities that include dystonia, hypokinesia, gait, and rigidity.

The pathophysiology of Mn neurotoxicity is complex and not well understood. The localization of Mn in the basal ganglia leads to the disruption of normal neurotransmission and results in subsequent neurodegeneration. The mechanistic basis of Mn toxicity is thought to be due to its deposition in the mitochondria, where it is reported to cause mitochondrial dysfunction by interfering in energy metabolism and increasing reactive oxygen species (ROS). Furthermore, it is reported that high levels of Mn may alter signaling pathways within the cell, as well as alter transcription and translation of particular proteins in neuronal and glial cells. The homeostasis of glutamate (GLU), the major excitatory neurotransmitter in the brain, is also documented to be greatly affected

by Mn. The excessive extracellular accumulation of GLU in manganism is hypothesized to be due to increased GLU release, and\or decreased extracellular rate of clearance. These changes are suggested to occur as a result of attenuated uptake of GLU by glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), which are the most predominant GLU transporters in the brain.

Providing mechanisms for mitochondrial protection is thought to prevent energy deficits, minimize ROS generation and protect the cells against apoptosis, which would minimize neurodegeneration. Recently, the use of creatine as a neuroprotective agent has emerged in both Parkinsonôs disease and Huntingtonôs disease. Current research supports the successful ability of creatine to protect against GLU excitotoxicity and mitochondrial dysfunction by buffering ATP depletion, preventing neuronal cell death, and activating antiapoptotic signaling pathways.

There are ***three primary goals for this study*****:** 1) examine the role of creatine treatment on the survival of Mn-exposed astrocytes; 2) examine the cause of Mn-induced GLU excitotoxicity in rat brain, and explore the effectiveness of creatine treatment in attenuating the effect of Mn; and 3) examine the effect of Mn on oxidative stress markers with or without creatine treatment. The **specific aims** for this study are:

1. **Examine the role of creatine treatment on the survival of Mn-exposed astrocytes.**

   Treatment of cortical astrocytes with creatine before and after Mn exposure. ***The working hypothesis for this aim*** *is that creatine will protect astrocytes against mitochondria-derived cell death by providing an exogenous substrate for ATP synthesis.*

2. **Examine the cause of Mn-induced GLU excitotoxicity in rat brain, and to explore the effectiveness of creatine treatment in attenuating the effect of Mn.** Twenty-one day old Sprague-Dawley rats were used to measure in vivo gene expression and protein levels of GLAST and GLT-1, and measure gene expression of brain creatine kinases in multiple brain regions as well as plasma creatine kinase activity. ***The working hypothesis for this aim*** *is that high levels of Mn may alter GLU transporters gene expression and proteins leading to GLU excitotoxicity and creatine will attenuate Mn effect by sparing energy deficiency in the cell and by increasing creatine kinases expression and activity.*
3. **Examine the effect of Mn on oxidative stress markers with or without creatine treatment.** Twenty-one day old Sprague-Dawley rats were used to measure in vivo protein levels of glutathione (GSH), and the gene expression of heme oxyogenase-1 (HMX-1). ***The working hypothesis for this aim*** *is that high levels of Mn may alter GSH protein levels and HMX-1 gene expression due to increased cellular oxidative stress and creatine will attenuate the neurotoxic effect of Mn.*

The general hypothesis of this study is that toxic levels of Mn may cause GLU excitotoxicity through alterations in GLU transporters. This excitotoxicity along with Mn-induced mitochondrial damage would subsequently result in increased ROS production and downstream events that lead to overall neurodegeneration. The use of creatine in Mn-exposure may help attenuate these changes by preventing energy metabolism impairment and ROS generation. Results from this study may elucidate the

role of Mn in neurodegenerative processes, and may provide insight into a new therapeutic agent.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Manganese (Mn) is an essential metal found in soil, water, and air. Although it exists in low concentrations within mammalian systems, Mn is important for normal physiological functioning.

Manganese has 11 oxidation states from -3 to +7. However, only Mn^{+2}, Mn^{+3}, and Mn^{+4} are found in mammals (Archibald and Tyree, 1987). In humans, Mn acts as a structural component for some metalloenzymes, these include arginase and superoxide dismutase (SOD). Manganese also acts as an activator of numerous enzymes, such as glutamine synthetase, which is responsible for the synthesis of glutamine from glutamate (GLU) (Keen et al., 2000). These roles make Mn essential for the metabolism of carbohydrates, lipids, and proteins. Moreover, Mn is important for growth, immune and skeletal development, and is vital for digestive and reproductive health.

Being an essential nutrient, Mn is obtained from the diet. The adequate intake (AI) of Mn has been established at 2.3 and 1.8 mg/day for adult men and women, respectively, with an increased intake for children and pregnant and lactating women (Aschner and Aschner 2005; ATSDR, 2009).

Manganese deficiency has not been reported in clinical settings; rather it has been seen in domestic animals and has been induced in lab animals causing metabolic

impairment, defective growth and abnormal skeletal development (Finley and Davis, 1999). On the other hand, excessive exposure to Mn causes a neurodegenerative disorder, known as manganism. This disorder, which resembles Parkinsonôs disease, is characterized by the accumulation of Mn in the brain causing symptoms such as, tremors, bradykinesia, dystonia, postural instability, gait abnormalities, insomnia, impotence, decreased memory and cognitive abilities, anxiety and symptoms of psychoticism (Mergler et al., 1994; Bowler et al., 2006).

Though cases of manganism have been predominantly reported in welders and miners, there is a growing concern of environmental exposure to toxic levels of Mn. This concern has been growing with the emerging use of Mn as a fuel additive in the form of methylcyclopentadienyl manganese tricarbonyl (MMT). Furthermore, highly populated areas that are located near Mn-emitting plants are also at risk of exposure to toxic levels of Mn via polluted air and water.

Manganese Exposure

Dietary Sources and Daily Requirements

Plants are an abundant source of Mn, specifically because Mn serves as the last electron accepter in the electron transport chain in photosynthesis. Whole grains, legumes, seeds and nuts are all Mn-rich plant sources.

Due to limitations on human research and the absence of clinical cases of Mn deficiency, no recommended dietary allowance has been established for Mn. However, in 1980 the National Research Council has determined the Estimated Safe and Adequate Daily Dietary Intake, which was set at 2.5-5 mg intake/day for adults (Friedman et al.,

1987). Moreover, due to the increasing concerns of Mn toxicity, the Environmental Protection Agency (EPA) has developed a dietary reference dose (RfD) of 0.14 $mg.kg^{-1}.d^{-1}$. Based on a body weight of 70 kg, the EPAôs RfD for dietary intake of Mn was set at 10 mg/day and for drinking water at 4.2 mg/day (Friedman et al., 1987; Finley and Davis, 1999). As mentioned previously, the most recent approach to determine safe intake levels of Mn was conducted by the National Academy of Sciences in 2001, which has established a newer AI of 2.3 and 1.8 mg Mn/day for adult males and females, respectively. However, during pregnancy and lactation, the need for Mn increases, indicating the importance of Mn for the development of the fetus and later the infant. Recommended AIs of Mn also vary for children depending on age and developmental stage (Aschner and Aschner, 2005).

Absorption

Based on studies that have used radio-labeled Mn (^{54}Mn), about only 1-5% of ingested Mn is absorbed in the gastrointestinal tract. There is a lot of controversy regarding the means of absorption. Some studies suggest an active transport mechanism, while others suggest a non-saturable passive diffusion (Aschner et al., 2005). However, it is known that Mn absorption in the gut can be influenced by multiple factors. One factor is the amount of Mn in the diet. Increased Mn in the diet leads to several adaptations by the intestine to decrease the absorption of the metal (Britton et al., 1966; Aschner et al., 2005), and causes other adaptations by the liver since the liver is responsible for regulating Mn turnover (Britton et al., 1966). Other factors that influence the absorption of Mn according to Davidsson et al. (1991) could include minerals (e.g. calcium),

phytates, and ascorbic acid. Nevertheless, iron (Fe) is considered one of the major effectors on Mn absorption and metabolism, where many human and animal studies throughout the years have demonstrated a possible interaction between Mn and Fe (Rossander-Hultén et al., 1991; Davis et al., 1992; Rodríguez-Matas et al., 1998). Finley et al. (1999) reported an inverse relationship between ferritin stores and Mn absorption in young women, where Fe deficiency enhances the absorption of Mn. Some researchers suggest competitive absorption and\or competitive transport (Aschner et al., 2005). Furthermore, gender difference in Mn absorption has been associated with the difference in ferritin stores among males and females (Finley et al., 1994).

Toxic Exposure to Manganese

Exposure to high concentrations of Mn leads to its subsequent accumulation in the brain, particularly in the basal ganglia. It has been found that Mn accumulates and damages the globus pallidus (GP), caudate-putamen (CP), substantia nigra (SN) and subthalamic nuclei (STN) of the basal ganglia leading to the extrapyramidal disorder, manganism (Yamada et al., 1986; Yu et al., 2003).

Toxic Exposure via Diet

There are no concerns of excessive exposure to Mn via oral ingestion in healthy individuals. Concerns only arise when the liver is either underdeveloped, such as in infancy, or when it is dysfunctional. Post absorption, Mn is delivered to the liver, which is responsible for regulating Mn excretion via bile (Papavasiliou et al., 1966). For infants, the risk arises with the use of milk formulas, which may have high levels of Mn (Aschner et al., 2005) or with the consumption of water from Mn-contaminated wells (Hafeman et

al. 2007; Bouchard et al., 2011). Multiple clinical cases of manganism have been observed in patients suffering of liver disease, particularly in chronic hepatic encephalopathy and cholestasis, where these patients developed neurological symptoms and had elevated blood Mn concentrations (Lazeyras et al., 2002; Burkhard et al., 2003). Symptoms of Mn overload have been observed in hospitalized patients receiving long-term total parenteral nutrition, where Mn bypasses the liverôs normal regulation process (Fell et al. 1996; Masumoto et al., 2001). This is seen especially in children, patients suffering of liver disease, and patients with intestinal obstruction disorders (Staunton and Phelan, 1995; Bertinet et al. 2000). Finally, the effect of Fe deficiency on absorption of Mn lays a risk on the worldôs huge Fe-deficient population. Finley and Davis (1999) suggested that the combination of a vegetarian diet with Fe deficiency could increase the risk of accumulating toxic levels of Mn in the body. Thus, given the world wide prevalence of Fe deficiency, the scenario of a large vulnerable population to Mn toxicity exists.

Toxic Exposure via Air and Water

1) Manganese as an Occupational Hazard

Exposure to high levels of airborne Mn and its subsequent accumulation in the brain has been known since 1837, when the first cases of manganism were described in ore crushers (Rodier, 1955). Welders, miners, smelters, and workers in steel and battery production factories, who are exposed daily to Mn fumes and dust, are at a great occupational risk of accumulating Mn in the brain and developing subsequent neurological abnormalities (Crossgrove and Zheng, 2004). According to the United

StatesôEPA, the risk of Mn airborne exposure increases in workers at Fe and steel production plants, power plants as well as coke ovens (U.S. EPA, TEACH, 2006). Thus, to prevent and decrease the occupational hazard of Mn, the Occupation Safety and Health Administration of the United States Department of Labor has established regulations to enforce a Permissible Exposure Limit of 5 mg/m^3 ceiling in general, construction and maritime industries. Furthermore, the National Institute of Occupational Safety and Health of the Center of Disease Control and Prevention, has set the limits of a Recommended Exposure Limit at 1 mg/m^3.

2) Manganese as an Environmental Hazard

Regardless of all regulations that have been established to prevent and decrease exposure at the occupational level, a great risk is posed on the public through environmental exposure. This risk is particular for those who dwell in areas that are near plants that release Mn-polluted air or water. Research supports the fact that there is an increased prevalence of Parkinsonôs disease-related symptoms in populations near Mn-producing industries (Lucchini et al., 2007; Catal´an-V´azquez et al., 2010). A study in Southern Ohio showed that living approximately 10 miles away from ferromanganese refinery led to subclinical impairment in postural balance due to exposure to low but *chronic* Mn levels in the air (Standridge et al., 2008). Another study in Mexico reported a significant correlation of changes in motor tests and airborne exposure to Mn in a population living near mining facilities (Catal´an-V´azquez et al., 2010). Furthermore, the effect of airborne environmental Mn exposure on children has revealed disturbing results of decreased intellectual abilities (Riojas-Rodriguez et al., 2010; Bouchard et al., 2011).

Manganese-contaminated drinking water, whether due to natural factors or to industrial waste or to the use of Mn-containing pesticides, has effects on birth outcomes, particularly causing low birth weight (Aschner et al., 2005). High levels of Mn in drinking water are also associated with decreased cognitive performance in children and to some extent in adults too (Bowler et al., 2006; Menezes-Filho et al., 2011).

Another rising concern of the publicôs environmental exposure is the use of Mn in the form of MMT as an antiknock agent in gasoline replacing the use of lead. The combustion of gasoline containing MMT would release Mn phosphates, Mn sulfates, and some Mn oxides into the air (Nelson et al., 2002). There is a lot of controversy when it comes to MMT and manganism. It has been suggested that the emission of Mn will contribute to the overall increase of Mn concentration in ambient air of urban areas (Davis et al., 1998). However, research reports that the amount of Mn released from vehicle emissions is too small to cause any clinical harm (Abbott, 1987). A study conducted in Toronto and Hamilton, two highly polluted cities that are heavily crowded with vehicle exhausts, have concluded that MMT in gasoline is not contributing to ambient air nor is it causing any neurological effects on subway workers, bus and taxi drivers, all who are in close proximity to exhaust air (Finkelstein and Jerrett, 2007). On the other hand, lab animals have shown neurological changes associated with MMT exposure (Salehi et al. 2006, Tapin et al, 2006). But it must be taken in account that the concentration of MMT used in these studies is at least 200 times more than its permissible concentration in the air of crowded cities that are known to use MMT (Taylor et al., 2006).

Transport of Manganese into the Brain

There are a number of theories regarding the entry of Mn into the brain. Some of these theories have been proved by *in vivo* and *in vitro* studies, while some theories remain questionable.

Inhalation: The Olfactory Neurons

The entry of particle matters through the olfactory neurons to the brain allows bypassing the tight regulation of the blood brain barrier (BBB). Elder et al. (2006) reported a 3.5 fold increase in Mn content of the olfactory bulb after 12 days exposure to Mn oxide, and a 2 fold increase in the lung tissue. The increase in Mn was also noted in other brain regions that included caudate-putamen, cortex, and cerebellum. Exposure of rats to $MnCl_2$ aerosols with one of the nostrils occluded revealed an increase in Mn levels in the olfactory bulb at the side of the open nostril compared to the occluded one (Brenneman et al., 2000). However, some researchers have reported that the travel of Mn through the olfactory will not necessarily lead to its accumulation in the caudate-putamen (CP), which is one of the brain regions that is usually targeted by Mn (Dorman et al., 2002). Thus, the fact that the olfactory nerve may serve as a direct route for Mn transport and deposition in the brain is somewhat debatable.

Inhalation: The Pulmonary Epithelium

It has been observed that the lung epithelia is involved in absorbing and accumulating neurotoxic Mn levels in the brain of both lab animals and humans (Roels et al., 1987; Dorman et al., 2005). The deposition and absorption of Mn particles in the lungs depend on the size, the density of the molecule, and solubilization by macrophages

(Roth, 2006). Once Mn^{+2} is trapped in the alveolar lymphoid fluid, it would be either transported into the blood via transferrin or via nonselective calcium (Ca^{+2}) channels but not by divalent metal transporter-1 (DMT-1) (Heilig et al., 2006).

The Blood Brain Barrier and the Choroid Plexus

Depending on its chemical form, Mn in the blood is found either bound to albumin (Mn^{+2}), transferrin (Mn^{+3}), or complexed to other molecules such as citrate and bicarbonate (Mn^{+2}) (Harris and Chen, 1994). While the transport of Mn across the BBB is thought to be temperature, energy, pH, [Fe], and [Na^{+}] -dependent (Fitsanakis et al., 2006), it mainly depends on plasma Mn concentration. At normal physiological concentrations, it is suggested that Mn passes through the capillary endothelium of the BBB by either endocytosis (when complexed to citrate or transferrin), or through particular carriers on the endothelium such as DMT-1 and ZIP-8 (Crossgrove et al., 2003; Corssgrove and Yokel, 2004; Aschner et al., 2007). Some studies suggest the presence of particular Ca^{+2}-dependant channels for Mn transport across the BBB (Corssgrove and Yokel, 2005**)**. At high plasma concentrations, Mn was found to cross the BBB-cerebral spinal fluid barrier via the choroids plexus (Murphy et al. 1991; Rabin et al. 1993). Wang and others (2008) reported a predominant role of transferrin rather than DMT-1 in the transport of Mn into the choroids plexus. However, more research is needed to reach to a conclusion regarding the transport of Mn into the brain.

Manganese Neurotoxicity

Manganese in the nervous tissue is normally found in low concentrations of approximately 1-2.9 εg/g dry weight, with the highest concentrations in the caudate-putamen (CP) and the globus pallidus (GP) (Prohaska et al., 1987; Bush et al., 1995). These regions, in addition to the substania nigra (SN), were reported to have significantly elevated Mn concentrations following Mn overload in nonhuman primates (Shinotoh et al., 1995; Guilarte et al., 2006). In Mn-exposed symptomatic and non-symptomatic workers, magnetic resonance imaging has shown an increase in the pallidal index, indicating high Mn deposition in the GP (Dietz et al., 2001; Kim et al., 1999). Furthermore, a 4 to 10 fold increase of Mn concentration was reported to be accompanied with significant neuronal atrophy in GP, and to a lesser extent in the CP (Maeda et al., 1997). Marked gliosis was also reported with an increase in astrocytic cellular count and characteristic Alzheimerôs Type II astrocytes in humans and monkeys (Maeda et al., 1997; Olanow et al., 1996). These alterations are associated with a disruption in the intracellular and extracellular neurotransmitter concentrations in the basal ganglia resulting in GLU excitotoxicity and decreased ɔ-aminobutyic acid (GABA) release from the CP and GP (Erikson and Aschner, 2003; Fitsanakis et al., 2006).

On the cellular level, toxic amounts of Mn tend to accumulate significantly in the mitochondria (Gavin et al., 1990; 1992), and to a lesser degree in the nuclei (Morello et al., 2008). The accumulation of Mn in the mitochondria in basal and toxic conditions may suggest a role of this organelle in Mn homeostasis in the cell similar to its role in regulating calcium (Ca^{+2}) (Gavin et al., 1999; Morello et al., 2008), and not merely due to

the localization of Mn-superoxide dismutase (SOD) in the matrix and inner membrane of the mitochondria as some suggested. The resulting mitochondrial dysfunction, particularly in astrocytes, is thought to contribute to the overall Mn neurotoxicity. Furthermore, studies have reported a role of Mn in inhibiting oxidative phosphorylation and generating reactive oxygen species (ROS), which results in cellular damage and energy metabolism impairment (Gavin et al., 1992; Milatovic et al., 2009).

Mechanisms of Manganese Neurotoxicity

Manganese induces neurotoxicity in the brain via multiple mechanisms. The accumulation of this metal in the basal ganglia, which is responsible for motor control, leads to impaired mitochondrial function, disrupted neurotransmission, increased oxidative stress, and subsequent cell death and neurodegeneration.

1. *Mitochondrial Dysfunction and Energy Deficiency*

The entry of Mn into the mitochondria occurs through the Ca^{+2} uniporter, where Mn tends to increase the uptake of Ca^{+2} (Gavin et al., 1992). High mitochondrial levels of Ca^{+2} inhibit Mn efflux resulting in its accumulation in the mitochondria, which in turn inhibits the exit of Ca^{+2} into the cytosol (Gavin et al., 1990; 1992). Therefore, Mn toxicity interferes with intracellular Ca^{+2} balance, and causes reduction in mitochondrial membrane potential (Milatovic et al., 2007), altogether leading to the subsequent opening of the mitochondrial permeability transition pore (mPTP) (Gunter et al., 1990).

Studies that have investigated the effect of Mn on energy metabolism have reported a significant decline in ATP levels in *in vitro* models of Mn-induced neurotoxicity (Brouillet et al., 1993; Zwingmann et al., 2003). The accumulation of Mn in

the mitochondria has been reported to reduce the efficacy of oxidative phosphorylation in producing ATP possibly due to Mn-induced reduction in the activity of complex I in the electron transport chain (Galvani et al., 1995; Yoon et al., 2011). Additionally, a decline in the activity of the Krebôs cycle enzymes and other mitochondria-related proteins has been reported in various Mn toxicity cell models (Zwingmann et al., 2003; Zhang et al., 2005). These changes not only cause energy failure but also contribute to the increased cellular oxidative stress and play a role in the induction of apoptosis.

Previous studies have also shown an increase in apoptosis and neuronal cell loss upon exposure to Mn (Hirata, 2002; Stanwood et al., 2009). In astrocytes, it has been documented that exposure to high levels of Mn result into the release of caspase-3 (Gonzalez et al., 2008; Yin et al., 2008), and the up-regulation of Bcl-2 proteins (Gonzalez et al., 2008). Furthermore, Prabhakaran and colleagues (2009) reported an increase in the release of cytochrome *c* and an up-regulation of BNIP-3 proteins in dopaminergic cell lines. The increase in these markers of apoptosis indicates a significant role of the dysfunctional mitochondria in initiating cell death following intoxication with Mn.

2. *Altered Neurotransmission*

Neurotransmission in the basal ganglia is a highly complex system that is made up of interconnected inhibitory and excitatory pathways acting in coordination with the cerebral cortex to regulate movement. The basal ganglia receive excitatory input via the thalamus from the cerebral cortex, and sends inhibitory signals back. The major neurotransmitters that are part of the basal ganglia-thalamus-cerebral cortex circuit

include the inhibitory neurotransmitters ɔ-aminobutyric acid (GABA) and dopamine (DA), as well as the brainôs major excitatory neurotransmitter glutamate (GLU).

As mentioned previously, Mn toxicity targets specific nuclei of the basal ganglia resulting in a movement disorder. It has been well-documented that Mn induces region-specific neurochemical changes by disrupting the synthesis, metabolism, and transport of GABA, DA, and GLU neurotransmitters, where these changes depend on severity and on the route of Mn exposure (Fitsanakis et al., 2006). Zwingmann and colleagues (2003) have shown alterations in the synthesis of GLU and GABA upon exposure to Mn. Nonetheless, Mn seems to cause changes in neurotransmitter concentrations by mainly affecting the transport system (Zwingmann et al., 2004; 2007). Previously published data report changes in DA neuronal D_1 and D_2 receptors (Fitsanakis et al., 2006), and report alterations in GABA transporter and receptors as well (Anderson et al., 2007).

Glutamate is tightly regulated in the CNS, where it must be kept at low concentrations in the extracellular fluid (ECF) to prevent excitotoxicity (Danbolt, 2001). The clearance of GLU from the ECF is a function of astrocytes, which express two high affinity Na^+-dependent transporters; these are glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1). Once GLU enters the astrocytes, it can be converted into Ŭ-ketoglutarate, or it can be amidated by glutamine synthetase in the presence of ammonia into glutamine, which is later released into the ECF for uptake by neurons (Danbolt, 2001). Moreover, studies have revealed that excessive exposure to Mn mediates excitotoxicity, where several mechanisms may be involved (Fitsanakis and Aschner, 2005).

One of the suggested mechanisms for excitotoxicity in manganism is decreased post-synaptic clearance of GLU by astrocytes (Hazell and Norenberg, 1997), where these cells tend to be the first cell type to accumulate Mn (Aschner et al., 1992). This high affinity towards Mn is thought to be due to the exclusive presence of glutamine synthetase in astrocytes, where this enzyme accounts for 80% of the total Mn in the brain. The decrease in the uptake of GLU is attributed to dysfunction in the GLU transporters. Some studies have proposed non-competitive binding of Mn to the transporters leading to GLU transport inhibition (Hazell and Norenberg, 1997). Meanwhile, it is also suggested that disrupted ion homeostasis, particularly Na^{+} and K^{+}, following Mn accumulation or the induction of ROS generation by Mn, may interfere the function of the GLU transporters (Hazell and Norenberg, 1997). Finally, it has been shown in previous studies that Mn disrupts gene and protein expression of GLAST and GLT-1 transporters, therefore suggesting Mn-induced genomic and post-translational modification of GLU transporters (Erikson and Aschner, 2002).

3. *Oxidative Stress*

Manganese is thought to have oxidant power similar to that of Fe in the Fenton reaction. Excess Mn^{+2} have been reported to induce formation of Mn^{+3}, where the latter is thought to have a higher capacity for oxidative stress induction (Kenten and Mann, 1957). In Mn neurotoxicity, Mn has been reported to contribute to the autoxidation of the CNS catecholamine such as DA (Lloyd, 1995), as well as fatty acids (Milatovic et al., 2007 and 2009). However, the increased oxidative stress in Mn overload is not limited to the pro-oxidant activity of Mn, but could also be secondary to other events taking place in

the cell, such as mitochondrial damage (Milatovic et al., 2007; 2009), ion imbalance (Scheuhammer and Cherian, 1981), and excess GLU (Trotti et al., 1998).

Although cells are equipped with defense mechanisms to scavenge ROS, Mn seems to alter antioxidant systems. For instance, levels of glutathione, a tripeptide antioxidant synthesized endogenously from GLU, cysteine, and glycine, were found to be altered upon airborne intoxication with Mn in rat and monkey brains (Taylor et al., 2006; Erikson et al., 2008). Liccione and Maines (1988) also reported changes in other related enzymes, such as glutathione peroxidase, glutathione reductase, as well as changes in catalase.

Heme oxygenase (HMX) is the rate limiting enzyme in the breakdown of heme into biliverdin, which is a process that involves the release of free Fe and carbon monoxide (CO) as byproducts (Ryter and Tyrrell, 2000). While there are 3 isoforms of HMX in the brain, HMX-1 is the major isoform that is expressed in neurodegeneration. The regulatory region of the HMX-1 gene is characterized with a binding site for nuclear factor kappa B (NFϖB), as well as metal response elements, cadmium response elements, and stress response elements, therefore the upregulation of HMX-1 can be induced by various stimuli that include heme, heavy metals, H_2O_2 and oxidized lipid products (Dennery et al., 2000). The overexpression of HMX-1 that is observed in multiple neurodegenerative diseases is thought to play a role in cellular defense against oxidative damage (Schipper et al., 2009). This is attributed mainly to the antioxidant and anti-inflammatory activity of byproducts of heme degradation, particularly bilirubin which results of biliveridin breakdown (Clark et al., 2000). While HMX-1 increased expression

is reported to have neuroportective effects against excitotoxicity and traumatic brain injury (Schipper et al., 2009), yet it is also found to induce cellular events that are harmful rather than protective. For instance, under certain conditions, the free Fe and CO that are released from the breakdown of heme contribute to increased oxidative stress and induce mitochondrial damage (Schipper et al., 2000). A recent study by Li and others (2011) investigated HMX-1 protein levels in PC12 cell model of Mn neurotoxicity and reported involvement of Mn in the activation of Nrf2 signaling pathway that leads to upregulation of HMX-1 gene expression. However, the role of HMX-1 and its expression in *in vivo* models of Mn neurotoxicity is unknown and is crucial for the understanding of the disrupted Fe metabolism that is reported in Mn toxicity.

4. *Other Mechanisms*

Manganese can also induce neurotoxicity by altering expression of various genes. This alteration occurs through the activation of inflammatory pathways as well as through the generation of free radicals that influence redox sensitive pathways (Chen et al., 2006). Moreover, Mn has been reported to disrupt Fe metabolism in the brain. Specifically, previous studies have reported an increase in Fe levels in the brain, therefore contributing in the neurotoxic events (Aschner et al., 2005; Chen et al., 2006).

Therapies for Manganese Neurotoxicity

The use of chelating agents has been the first therapy described for treating manganism (Humphreys, 1946). These agents work by binding metals, thereafter increasing their excretion rate and leading to accelerated metal removal. Calcium disodium versenate ($CaNa_2EDTA$) is a chelating agent that has been used in treating

manganism (Herrero Hernandez et al., 2006; Jiang et al., 2006). Studies have found $CaNa_2EDTA$ to be effective in decreasing Mn blood levels by increasing urinary excretion (Jiang et al., 2006). Although research shows $CaNa_2EDTA$ to be a successful therapy for acute exposure to Mn, this might not be the case in chronic exposure, where $CaNa_2EDTA$ or any other chelating agents have no effect on restoring the integrity of the damaged tissue (Cook et al., 1974; Crossgrove and Zheng, 2004). Moreover, the majority of chelating agents including $CaNa_2EDTA$ chelate other trace metals, such as Fe and Cu leading to subsequent deficiencies. On the other hand, *p*ara-aminosalicylic acid (PAS), a drug used in treating tuberculosis, can act as a chelating agent through increasing Mn excretion via feces (Tandon et al., 1978). *Para*-aminosalicylic acid has been recently used in treating Mn and has yielded an overall improvement in neurological performance in cases of chronic Mn exposure (Ky et al., 1992; Jiang et al., 2006), which could be likely due to removal of Mn. A recently published study by Zheng et al. (2009) on rats receiving intraperitoneal injections of $MnCl_2$, reported PAS to be effective in removing Mn without inducing Fe deficiency, however the exposure route used in this study is not a common route for excessive exposure to Mn. Furthermore, only few papers in the literature address the use of PAS in manganism, and hence more research is needed.

Levodopa (L-dopa), a precursor of DA, is a drug used for treating Parkinsonôs disease. L-dopa crosses the blood brain barrier and compensates the loss of DA in the brain. Clinical cases of manganism have yielded different outcomes with the use of L-dopa (Huang et al., 1993; Kenangil et al., 2006; Lu et al., 1994). It has been reported that long term L-dopa treatment contributes to the severity of the disease rather than

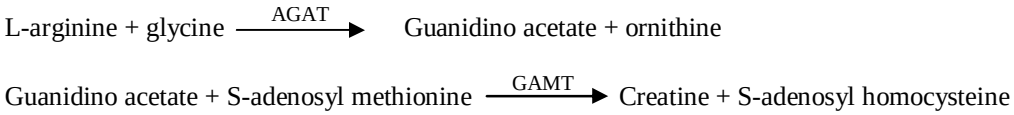
alleviating symptoms, where in vivo studies have shown a significant increase in auto-oxidation of L-dopa when co-administered with Mn (Serra et al., 2000). Thus, the use of L-dopa holds no promises for treating Mn neurotoxicity.

Recent in vitro and in vivo approaches for treating toxicity of Mn have targeted the use of antioxidants, where they are hypothesized to enhance cellular response to Mn-induced oxidative stress. Studies using dopaminergic cell lines have shown a protective role of N-acetyl-L-cysteine against Mn-induced apoptosis (Kitazawa et al., 2002; Zhang et al., 2009; Yoon et al., 2011). However, recent data from our lab show that treating Mn-exposed rats with N-acetyl-L-cysteine did not yield any significant changes in profiles of oxidative stress markers, while treatment with epigallocatechin gallate, the prominent antioxidant found in green tea extract, had only limited benefits. Silymarin, an antioxidant flavonoid, has been found to attenuate Mn-induced cell death as well as improve endogenous antioxidant activity (Chtourou et al., 2010; 2011). Other recent therapies that are being tested in vitro include: cyclopentanone prostaglandin, an antitumor drug that is found to impose neuroprotective properties (Furuta et al., 2007; Shibata et al., 2009), as well as riluzole, which is a drug used in amyotrophic lateral sclerosis (ALS) to attenuate GLU excitotoxicity (Deng et al., 2009).

While currently available therapies are not efficiently effective in curing and reversing symptoms of manganism, and while new emerging therapies tend to target only one mechanistic aspect of Mn neurotoxicity, there is a need for finding a therapy that would restore altered biochemical parameters and enhance cellular defense against Mn toxicity.

Creatine

Creatine or methylguanidino-acetic acid is a guanidino compound that can be synthesized endogenously from arginine, methionine, and glycine. The synthesis of creatine requires a two-step reaction that involves two enzymes; arginine:glycine amidino transferase (AGAT) and S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT).



These enzymes are only found in the liver, as well as in the kidney, pancreas, testes, and in the brain (Walker, 1979; Persky and Brazeau, 2001; Braissant et al., 2001). Creatine can also be obtained from the diet, where it is found abundantly in red meats and in fish (Balsom et al., 1994).

Creatine is either synthesized endogenously depending on availability of AGAT and GAMT enzymes in the tissue or it can enter the cell from the blood circulation via Na^+ and Cl^- dependent creatine transporter-1 (CRT-1), where creatine is usually concentrated in tissues with high fluctuating energy demands, such as the skeletal muscles, the heart, and the brain, due to its critical role in energy homeostasis (Wallimann et al., 1992). Creatine kinases (CKs) are magnesium-dependent phosphotransferases that are found in different tissue-specific isoforms. These enzymes catalyze the hydrolysis of ATP, where they help dephosphorylate ATP into ADP allowing creatine to bind the phosphate group and form phosophcreatine (PCr), which is a storage form of energy. When there is a sudden demand for energy in the cell, CKs with

their low-threshold ADP sensor facilitate the reverse reaction, leading to the release of creatine and ATP. It is noteworthy that this energy system is the first to respond to energy needs within the cell, taking less time to release ATP than other energy systems such as glycolysis and oxidative phosphorylation (Walliman et al., 1992).

Creatine in the Brain

In the last few years, creatine has been targeted in neurodegenerative diseases due to its potential neuroprotective role in the brain. The brain is supplied with creatine either from the blood circulation, where creatine is capable of crossing the blood brain barrier through CRT-1, or it can be supplied by brain creatine synthesis. Recent data report expression of AGAT and GAMT in neurons, oligodendrocytes, and in astrocytes. It was suggested that the synthesis of brain creatine is restricted in astrocytes; however, a recent study by Braissant et al. (2011) has revealed that various cells in the CNS do not co-express the two enzymes, which is indicative of the importance of neuro-astroglial interactions for the synthesis of creatine. Additionally, CRT-1 has been reported to be expressed in both neurons and oligodendrocytes; however it is unclear if astrocytes express this transporter (Braissant et al., 2001; Möller and Hamprecht, 1989).

Creatine in Neurodegenerative Disorders

Recently, approaches for the use of creatine as therapy in neurodegenerative diseases has emerged, where studies have been moved from the bench to patient bedsides, particularly in Huntingtonôs disease, amyotrophic lateral sclerosis (ALS), and in Parkinsonôs disease. Animal models of Huntington's disease, an inherited neurodegenerative disease characterized with striatal excitatory lesions and oxidative

damage, have yielded positive results when treated with creatine. The results of these studies have shown enhanced animal survival, decreased rate of degeneration, improved motor performance, and increased ATP brain levels (Ferrante et al., 2000; Andreassen et al., 2001a; Dedeoglu et al., 2003). Moving creatine into the clinical setting, a trial by Bender and others (2005) has reported a decrease in GLU excitotoxicity in Huntingtonôs patients receiving 20 g/day for the first 5 days, and 6 g/day for the reminder of the study (10 weeks). In ALS, which is a neurodegenerative disorder characterized by mitochondrial dysfunction and cortical neuronal loss, creatine improved motor function in mice (Klivenyi et al., 1999), and decreased extracellular accumulation of GLU (Andreassen et al., 2001b). Moreover, in a phase I clinical trial of creatine, treatment with 15 g creatine/day caused an increase in brain levels of creatine, and a decrease in GLU concentrations (Atassi et al., 2010). Creatine treatment in Parkinsonôs disease seems to show promise in delaying the progression of the disease. As mentioned previously, Parkinsonôs disease resembles manganism; however Parkinsonôs disease is characterized with nigral neuronal damage rather than pallidal damage as observed in Mn neurotoxicity. Creatine has been shown to cause increased survival rate of cell models of Parkinsonôs disease (Andres et al., 2005a; 2005b), and decrease in vivo biochemical alterations of key enzymes used as markers of oxidative stress (Matthews et al., 1999). In a clinical trial, creatine has led to improvement in overall patient mood and decreased required L-dopa dosage (Bender et al., 2006). Recently in 2007, the National Institute of Health (NIH) has announced a phase III clinical trial for the use of creatine in treating Parkinsonôs disease (Bloom, 2007). Finally, more studies are conducted to determine a

possible role of creatine in treating ischemia (Zhu et al., 2004) and in improving cognition in elderly (Rawson and Venezia, 2011).

Neuroprotective Mechanisms of Creatine

The role of creatine in maintaining ATP\ADP ratio at equilibrium in the cell, and preventing energy deprivation during rapid bouts of neuroexcitation are thought to be the major reasons behind its neuroprotective ability. Furthermore, the constant supply of ADP into the mitochondrial matrix that follows the phosphorylation of creatine by mitochondrial creatine kinase (CK) is suggested to participate in decreasing ROS generation from oxidative phosphorylation (Meyer et al., 2006), where the latter is known to be the major source of ROS production in the cell (Chance et al., 1979).

Besides its role in energy balance, creatine is thought to contribute to the regulation of neurotransmission. A study by Xu et al. (1996) has reported a PCr-dependent uptake of GLU in synaptic vesicles in addition to the ATP-dependent uptake. Moreover, studies in models of Huntingtonôs and ALS have shown creatine supplementation to decrease GLU excitotoxicity (Bender et al., 2005, Andreassen et al., 2001b). This attenuation of excitotoxicity is thought to be due to PCr providing energy that causes the restoration of Na^+ gradient, which is needed for GLU active transport (Brustovetsky et al., 2001). Additionally, creatine has been found to alter the activity of GLU decarboxylase, where this enzyme is involved in the synthesis of GABA (Schultheiss et al., 1990; Peña-Altamira et al., 2005).

One of the most controversial neuroprotective properties of creatine is its ability to prevent the formation of the mPTP, therefore delaying induction of apoptosis. The

octameric structure of the mitochondrial CK allows it to bind phospholipids in both the inner and outer mitochondrial membranes forming contact sites between these two membranes and allowing for mechanical stabilization (Speer et al., 2005). Furthermore, mitochondrial CK is found to be coupled with adenine nucleotide translocator (ANT), which participates in the events of the opening of the mPTP. A study conducted by Dolder and colleagues (2003) has reported an inhibition in the mPTP formation in isolated liver mitochondria expressing mitochondrial CK upon treatment with 10 mM creatine. However, this inhibition seems to be rather moderate as described in an in vitro study (Brustovetsky et al., 2001).

Other suggested neuroprotective mechanisms of creatine include a possible antioxidant activity, where creatine has been reported to protect RNA against oxidative damage (Fimognari et al. 2009). Moreover, creatine is found to be involved in inducing signaling pathways within the cell such as NFϖB signaling pathway, which may explain the increase in survival rate in culture and animal studies (Juravleva et al., 2005). Finally, creatine has been found to play a role in cellular neuronal cell differentiation and development (Andres et al., 2005; Ducray et al., 2007), which is indicative of roles far beyond energy maintenance.

Conclusion

The exposure of vulnerable populations to toxic levels of Mn, whether occupational, environmental, or dietary, indicates a need to find a preventative approach to protect these populations, and to find a cure that would grant better prognosis for those who develop manganism. While Mn induces its toxicity through a number of

mechanisms that include oxidative stress, disrupted energy metabolism and neurotransmission, there is a constant need to find a therapeutic agent that would target all these mechanisms at once. Creatine, being considered for phase III clinical trial in Parkinsonôs disease, might be such a therapeutic agent to cure or help prevent manganism. While creatine may not be able to chelate the metal, its ability to balance energy metabolism in the cell, and to protect against oxidative stress may bring forth the long awaited cure for manganism.

CHAPTER III

EVALUATION OF CREATINE AS A NEUROPROTECTIVE AGENT AGAINST MANGANESE-INDUCED NEUROTOXICITY IN ASTROCYTES

Abstract

Manganism is a Parkinsonôs-like neurodegenerative disorder that results from toxic accumulation of manganese (Mn) in the brain. It is well documented that astrocytes, which play a dynamic role in brain ion homeostasis and energy metabolism, are vulnerable to cytotoxicity caused by excessive Mn exposure. Currently, there are no known effective therapies to treat manganism; however, recently the use of creatine as a neuroprotective agent has emerged. Since astrocytes are the primary cells to respond to neurotoxins, we hypothesized that treating astrocytes with creatine prior to and after Mn exposure would decrease Mn-induced cytotoxicity and result in modulation of biomarkers of neurotoxicity that include creatine transporter (CRT-1), glutamate-aspartate transporter (GLAST), and glutathione peroxidase (GPx). Primary astrocytes were cultured and divided into five groups: controls (CN), Mn group (300 ε M $MnCl_2$ for 24 hours), creatine group (1 mM creatine monohydrate for 24 hours), Mn followed by creatine treatment group (MnCr), and creatine treatment followed by Mn exposure group (CrMn). Results show that cellular viability was significantly decreased in the Mn group ($p < 0.05$), and that creatine attenuated the toxic effects of Mn in the MnCr and CrMn

groups. The concentration of Mn was significantly elevated in Mn and CrMn groups, but not in MnCr group. A significant elevation in GLAST gene expression was seen in CrMn compared to CN, and GPx gene expression was significantly decreased in MnCr and CrMn groups, however there were no significant changes in CRT-1 gene expression due to either Mn or creatine exposures. In conclusion, it appears that the application of an exogenous source of creatine prior to or after Mn exposure may impose some sort of neuroprotection in primary cultured astrocytes.

Introduction

Manganese (Mn) is an essential metal that is needed for various physiological processes. However, excessive exposure to this metal may result in an extrapyramidal syndrome that resembles Parkinsonôs disease. Manganese concentration in different brain tissue has been found to exceed 200 εM in monkey and rat models of Mn-induced neurotoxicity (Roels et al. 1997; Ingersoll et al., 1999; Lai et al., 1999). It is well documented in the literature that Mn exerts its neurotoxic effects through inducing oxidative stress (Taylor et al., 2006; Milatovic et al., 2007), and through disrupting normal neurotransmission (Fitsanakis et al., 2006) and energy metabolism (Zwingmann et al., 2003).

Astrocytes are the housekeeping cells of the CNS; they are responsible for maintaining a balanced extracellular environment, which includes the clearance and recycling of the major excitatory neurotransmitter, glutamate. Two high affinity Na^+-dependent protein transporters are expressed on the cell surface of astrocytes for the uptake of glutamate. These are glutamate-aspartate transporter (GLAST) and glutamate

transporter-1 (GLT-1). Astrocytes are also responsible for the uptake of Mn, particularly due to glutamine synthetase enzyme, which is only found in astrocytes and is involved in the amination of glutamate to form glutamine, a key process in brain ammonia metabolism. In Mn neurotoxicity, astrocytes undergo alterations that lead to energy metabolism failure (Zwingmann et al., 2003), and changes in glutamate uptake (Hazell and Norenberg, 1997; Erikson and Aschner, 2002), which subsequently leads to excess extracellular fluid glutamate and excitotoxicity. Additionally, excessive exposure to Mn is reported to increase the generation of reactive oxygen species (ROS) and therefore contribute to cytotoxicity in astrocytes, where endogenous antioxidants fall short in protecting against these oxidative stressors, as their levels seem to be altered (Taylor et al., 2006). For instance glutathione, a thiol antioxidant that scavenges free radicals in the presence of glutathione peroxidase enzyme (GPx), has been found to decrease in neurons intoxicated with Mn (Zwingmann et al., 2003).

Recently, the use of creatine in neurodegenerative disorders has emerged. Creatine has shown neuroprotection in models of Huntingtonôs disease (HD) (Ferrante et al., 2000; Dedeoglu et al., 2003), amyotrophic lateral sclerosis (ALS) (Klivenyi et al., 1999), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of Parkinsonôs disease (Matthews et al., 1999). Creatine is a guanidino compound that is found in cells of high energy-fluctuating needs such as the skeletal muscles, brain, and heart. Creatine is either synthesized endogenously by the liver, and kidney or it is supplied through diet by consuming fish and fresh meat (Balsom et al., 1994). Synthesis of creatine in the CNS has been found to occur in both neuronal and glial cells, where expression of *S*-adenosyl-

L -methionine:*N*-guanidinoacetate methyltransferase (GAMT) and L-arginine:glycine amidinotransferase (AGAT), the enzymes required for the synthesis of creatine from L-arginine, has been documented (Dringen et al., 1998; Braissant et al., 2001). Creatine is used mainly as an energy buffer along with phosphocreatine (PCr). The transfer of a phosphate group from ATP to creatine to form PCr and ADP is carried out by both mitochondrial and cytosolic creatine kinases (CKs). When there is an increased demand for energy in the cell, CK transfers the phosphate group from PCr into ADP, to make more ATP readily available for the biological processes within the cell. Creatine is transported into the blood brain barrier through a creatine transporter (CRT-1). Several studies have reported the expression of CRT-1 in neurons and oligodendrocytes, but not in astrocytes (Braissant et al., 2001).

Given the similarities between manganism and Parkinsonôs disease, the promising neuroprotection of creatine on Parkinsonôs disease by slowing down the disease progression as observed in clinical trials (NINDS NET-PD Investigators, 2006; 2008) may also be seen in models of Mn neurotoxicity, especially with the lack of an effective therapy for the latter. Therefore, it is important to investigate the use of creatine as a therapeutic agent for treating Mn-induced neurotoxicity and identify the underlying mechanisms of action. The purpose of this study was to evaluate the use of creatine in Mn neurotoxicity in astrocytes, and identify changes in gene expression of CRT-1, GLAST, and GPx genes.

Materials and Methods

Cell Cultures

Primary cortical astrocytes were obtained from GIBCO (Invitrogen, Carlsbad, CA). Briefly, the cells were isolated under sterile condition from the cortices of fetal Sprague-Dawley rats (gestation day 19), and grown in astrocyte growth medium (85% Dulbeccoôs Modified Eagle medium (DMEM) containing 4.5 g/L glucose, and 15% Fetal Bovine Serum) before being cryopreserved and shipped in 90% astrocyte growth medium plus 10% DMSO. Afterwards, astrocytes pellet was thawed on ice, and cells were plated at 1 x 10^5 cell/mL density in a 6-well plate. Cell cultures were grown in DMEM containing 15% Fetal Bovine Serum, and maintained at 37 °C in humidified 95% air with 5% CO_2. Media was changed 2-3 times per week for 3 weeks until >95% confluence was achieved. Cells were divided into 5 groups treated as follows: (1) 24 hours 300 εM $MnCl_2$ (Mn group), (2) 24 hours 1 mM creatine (Cr group), (3) 24 hours of 300 εM $MnCl_2$, followed by 24 hours 1 mM creatine treatment (MnCr group), (4) 24 hours of 1 mM creatine, followed by 24 hours of 300 εM $MnCl_2$ (CrMn group), and (5) control group.

MTT Viability Assay

Cell viability was measured by using 3-(4, 5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, Saint Louis, MO). After the end point of each treatment, the cell cultures were washed in PBS, and 1.5 ml DMEM media containing 150 εl MTT dissolved in DMEM (5 mg/ml) was added for each well. Cell cultures were then incubated at 37 °C in 5% CO_2 incubator for 2 hours. Following

the incubation period, a 1.5 ml of MTT solubilization solution was added per well to dissolve formazan crystals. Thereafter, absorbance was measured with a plate reader wavelength of 550 nm, using a reference of 630 nm.

Metals

Astrocytes harvested in PBS were sonicated and Mn, Fe, and Cu concentrations were measured using graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). 300 εl of cell homogenate was digested in 100 εl of ultrapure nitric acid for 48-72 hours in a sand bath at 60° C. An additional dilution with 2% nitric acid was made prior to the analysis. Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 εg Mn/g; 184 εg Fe/g; 80 εg Cu/g) was digested in ultrapure nitric acid and used as an internal standard for analysis (final concentration 5 εg Mn/L; 92 εg Fe/L; 40 εg Cu/L).

RT-PCR

At the end of the treatment periods, culture media was removed, and 1 ml of denaturation solution was added to each well. Cells were then harvested and sonicated. Messenger-RNA was isolated from the cells homogenates using ToTALLY RNAÊ kit (Ambion Inc., Austin, TX) following the manufacturerôs instructions. Prior to cDNA synthesis, RNA concentration and purity were determined using A260:A280 ratio produced by NanoDrop spectrophotometry (Thermo Scientific, Delaware, MD). For cDNA synthesis, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used as indicated in the manufacturerôs instructions guide. TaqMan® Gene Expression Assays were purchased to probe for GLAST, Creatine

transporter, and GPx gene expression, in conjunction with Universal PCR Master Mix (Applied Biosystems, Foster City CA). Triplicate aliquots of cDNA were analyzed on 96-well plates using the expression assays for the genes of interest. All gene expression values were normalized to ß-actin expression value from the same sample on the same plate and reported as percent of control.

Statistics

Data were analyzed using SPSS version 14 for windows (Chicago, IL). The analysis included using a two-way analysis of variance and two-tailed t-tests to detect differences among groups. Dunnettôs post hoc analysis was used to determine difference from controls when $p < 0.05$. Outliers were removed via boxplot with ± 2 standard deviation criteria.To identify relationships between Mn concentrations and mRNA levels, two-tailed Pearsonôs correlations analyses were conducted.

Results

Metals Concentrations

Mn was significantly elevated in Mn and CrMn groups ($p<0.05$) when compared to controls, whereas Cr and MnCr treatment did not significantly increase Mn levels (Table 3-1). Fe concentrations were significantly increased five-fold in CrMn when compared to control ($p<0.05$) but no other treatment caused elevated Fe levels. No significant treatment effects were observed with cellular Cu concentrations (Table 3-1).

Table 3-1: Metal Concentrations
Manganese, iron, and copper concentrations in cultured astrocytes expressed as ε g [Metal]/ mg protein ± S.E.M.

Group	[Mn]	[Fe]	[Cu]
Control	0.01998 ± 0.00379	0.07217 ±0.01330	0.02270 ± 0.00260
Mn only	2.35844 ± 0.04434*	0.04751 ± 0.00519	0.01619 ± 0.00076
Creatine only	0.02259 ± 0.00207	0.07272 ± 0.00977	0.01643 ± 0.00093
MnCr	0.23189 ± 0.02215	0.08862 ± 0.02065	0.02531 ± 0.00356
CrMn	2.47759 ± 0.31734*	0.37521 ± 0.15974*	0.01749 ± 0.00170

**Statistically Significant at p < 0.05 Dunnet's post hoc test.*

MTT Viability Assay

The viability of astrocytes treated with Mn alone decreased significantly by ~ 45% when compared to control (p<0.05) (Figure 3-1). Additionally, Mn group and MnCr group were significantly decreased when compared to Cr group, which was ~ 20% higher than controls. However, CrMn group, although lower than controls by ~ 20%, was not statistically significantly different from controls.

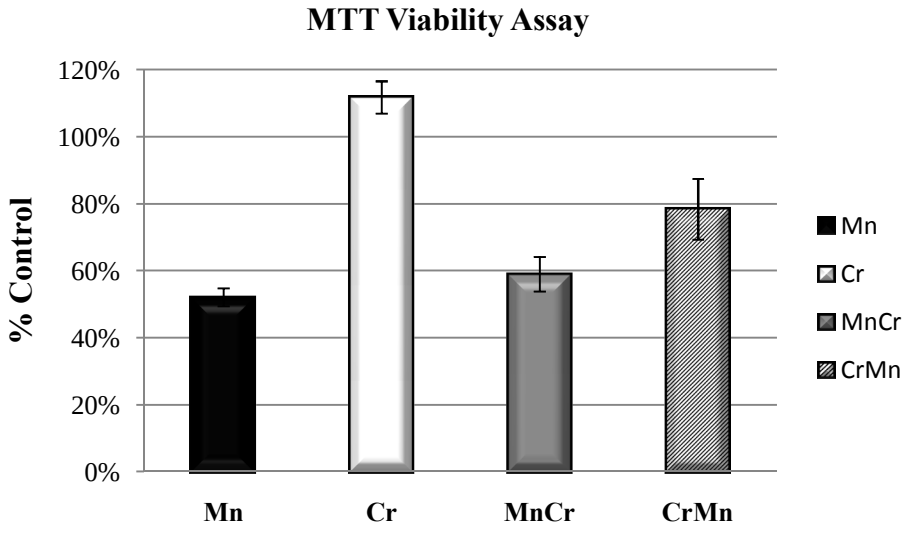


Figure 3-1: MTT Viability Assay. Mean group viability expressed as percent mean control in Mn, Creatine, MnCr, and CrMn primary astrocyte cultures. Mn exposure significantly decreased viability in Mn group. Creatine group seemed to have a slight increase in viability. MnCr and CrMn groupsô viability was elevated compared to manganese group, where the increase in viability was higher in CrMn than in MnCr. **Statistically Significant at p < 0.05 Dunnet's post hoc test.*

GPx Gene Expression

A significant decrease in GPx gene expression was observed in MnCr and CrMn group ($p < 0.05$) when compared to controls (Figure 3-2). However, this decrease along with the decrease in Mn group was not significantly correlated with Mn concentrations.

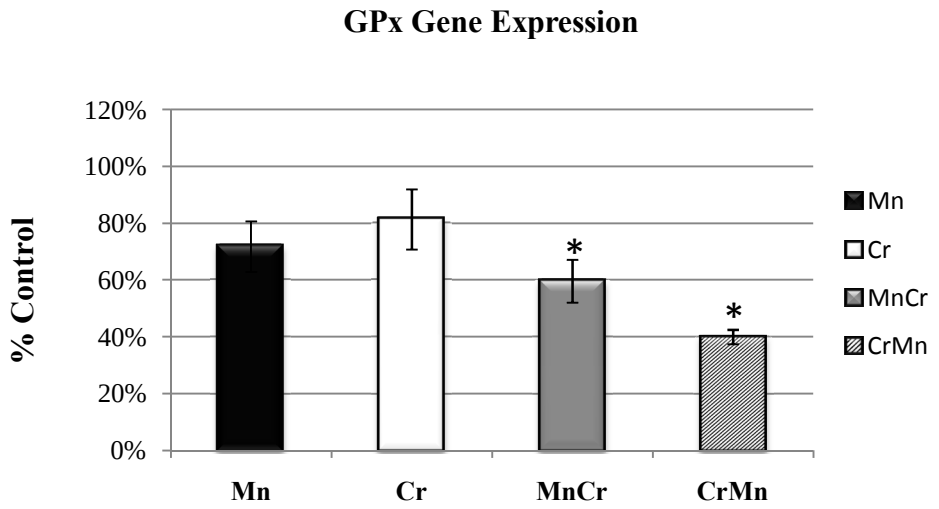


Figure 3-2: GPx Gene Expression. Effect of Mn and creatine on glutathione peroxidase (GPx) gene expression in cultured astrocytes. Mn attenuated the gene expression of GPx in Mn, MnCr, and CrMn groups, where the decrease in the Mn-exposed Cr-treated astrocytes was statistically significant. **p<0.05 according to Dunnett's post hoc analysis.*

GLAST Gene Expression

Although Mn group has shown a 50% decrease in GLAST gene expression when compared to control, this change was statistically not significant (Figure 3-3). However, a statistically significant increase was observed in CrMn group ($p < 0.05$). This increase did not show a correlation with Mn concentration.

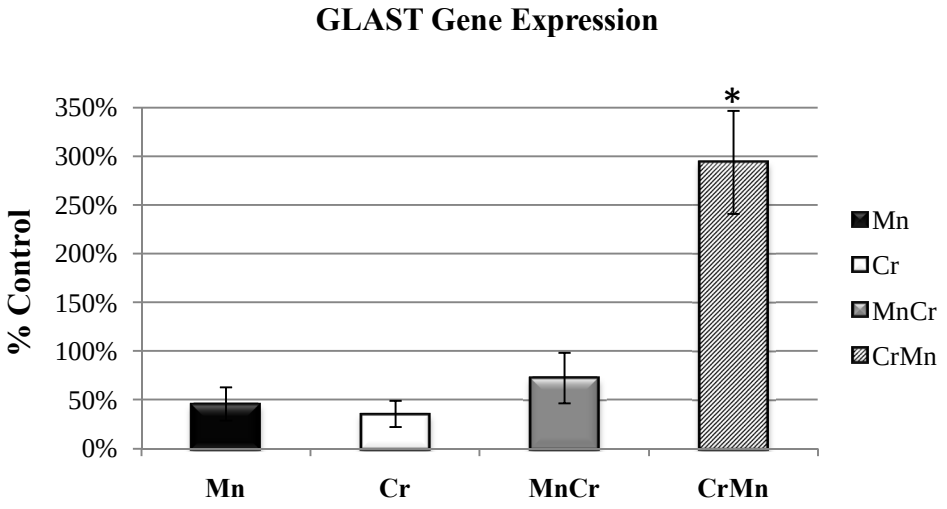


Figure 3-3: GLAST Gene Expression. Effect of Mn and creatine on glutamate:aspartate transporter (GLAST) gene expression. Mn caused a 50% non-significant decrease in GLAST gene expression when compared to controls. While exposure of astrocytes to Mn after being treated with creatine yielded a 3 fold increase in GLAST gene expression. **p<0.05 according to Dunnett's post hoc analysis.*

Creatine Transporter Gene Expression

Results show an expression of CRT-1 in astrocytes. However, no significant changes were observed in CRT-1 gene expression upon exposure to Mn or treatment with creatine (Figure 3-4).

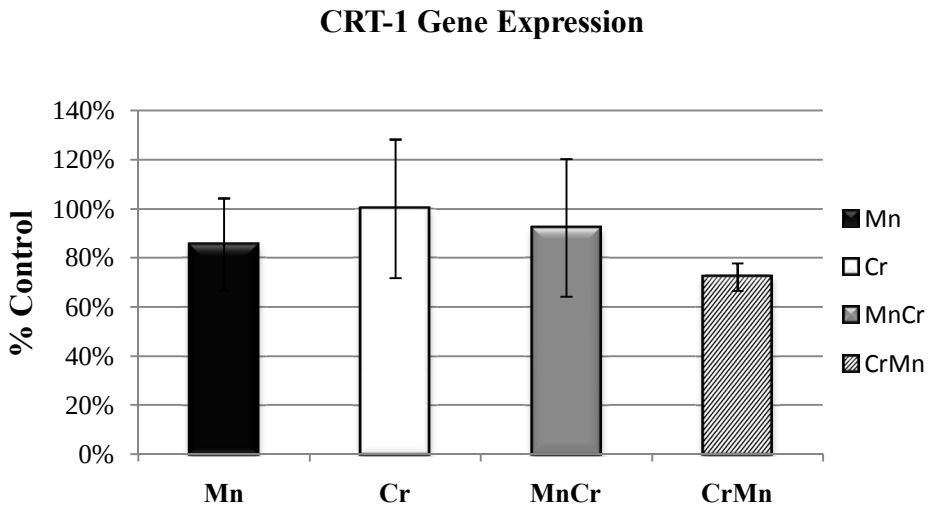


Figure 3-4: CRT-1 Gene Expression. Effect of Mn and creatine on creatine transporter gene expression. Mn or creatine did not cause any changes in creatine transporter gene expression.

Discussion

In this study we demonstrate the role of creatine in protecting against Mn cytotoxicity in astrocytes. Several novel findings emerged from this study. First, exposure of astrocytes to 300 ε M of $MnCl_2$ led to decreased viability, which corroborates previous studies using both lactate dehydrogenase assay and MTT assay (Yin et al., 2008), but creatine treatment prevented this loss of viability. Second, expression of GLAST trended to decrease in Mn, Cr, and MnCr group potentially affecting the uptake of glutamate; however there was a significant increase observed in CrMn group. Finally, GPx gene expression was decreased in all Mn exposed groups, which may lead to decreased GPx protein synthesis and thereafter disrupt glutathione (GSH) antioxidant cycle. Third, although CRT-1 was neither upregulated nor downregulated due to Mn-exposure, we report detectable levels of CRT-1 mRNA in primary cultured astrocytes.

The 40% decrease in viability in the Mn group is similar to the decreased viability of other CNS cells exposed to Mn. Studies of rat brain endothelial cells (dos Santos et al., 2010), dopaminergic cells (Prabhakaran et al., 2009), PC12 cells (Reaney and Smith, 2005), and astrocytoma and neuroblastoma cell lines (Dukhande et al., 2006) all have demonstrated a decrease in viability upon treatment with at least 200 ε M $MnCl_2$, meanwhile the effect of creatine on astrocytic viability has not been examined. In MPTP-exposed dopaminergic neurons, a 7 day treatment with 5 mM creatine was reported to increase neuronal viability by 1.32 folds when compared to controls (Andres et al., 2005a). In a rat model of ALS, creatine decreased GABAergic neuronal death markers significantly, while in mice an overall increase in animal survival was observed (Peña-

Altamira et al., 2005). Another study reported increased differentiation rather than viability in striatal GABAenrgic neurons in models of HD following 5 mM creatine treatment (Andres et al., 2005b). We repeated the experiment twice for validation, and similar results were yielded. Despite the inconsistent results in creatine treated Mn-exposed groups, overall the viability of CrMn group was always higher than the viability of MnCr group regardless of the higher Mn concentrations measured in CrMn cells. This difference may indicate that pre-treatment with creatine exerts some protection against Mn neurotoxicity. However, for the MnCr group, it is possible that Mn has attenuated the effect of creatine. A recent study shows some evidence of this possible attenuation since Mn exposure led to a depletion of ATP (an important substrate for creatine biology) in astrocytes (Milatovic et al., 2007). Additionally, the creatine dosage used (1 mM) and exposure time (24 hours) might not have been sufficient enough to produce statistically significant changes.

In situ hybridization has previously reported lack of CRT-1 gene expression in astrocytes (Braissant et al., 2001); however, using RT-PCR, we showed expression of CRT-1 in primary cultures astrocytes. It is possible that the in situ hybridization used in previous studies was not sensitive enough to detect mRNA levels; furthermore other studies that have treated cultured astrocytes with creatine have showed a response to creatine treatment. For instance, treatment of astrocytes with 5 mM of creatine attenuated the uptake of L-arginine (the rate limiting amino acid in creatine biosynthesis) by approximately 15% when compared to controls (Schmidlin and Wiesinger, 1994), and treatment of astrocytes with 25 mM of creatine delayed the toxic effects of 3-

nitropropionic acid (Deshpande et al., 1997). These studies are indicative of the uptake of creatine by astrocytes.

The concentration of the excitatory neurotransmitter glutamate is tightly regulated and maintained at low concentrations in the extracellular fluid of the CNS to prevent excitotoxicity (Rothstein et al., 1996). About 80% of the glutamate released in the synaptic space is taken up by astrocytes, where it is either committed to the glutamate-glutamine cycle, or itôs committed to the TCA cycle (Danbolt, 2001). GLAST, one of the major glutamate transporters, is expressed in cultured astrocytes (Kondo et al., 1995). Changes in this transporter are thought to be the cause of decreased uptake of glutamate in Mn neurotoxicity, which eventually leads to excitotoxicity that is thought to contribute to Mn toxic effects. Previous studies have attributed the decrease in glutamate uptake to a possible binding of Mn to GLAST (Mutkus et al., 2005), or to a possible disruption in the electrochemical gradient that is related to GLAST regulation (Hazell and Norenberg, 1997). Meanwhile, the decreased gene expression of GLAST upon Mn exposure could be another possibility for the decreased uptake of glutamate, where it has been previously reported that an overnight exposure to 500 εM of Mn decreases GLAST gene expression significantly (Erikson and Aschner, 2002). In another study, where Chinese hamster ovary cells were transfected with GLAST, exposure to 500 and 1000 εM of Mn for 18 hours also resulted in a significant decrease in GLAST gene expression (Mutkus et al., 2005). In this study, exposure of astrocytes to 300 εM Mn for 24 hours (Mn and MnCr groups) has shown a non-significant decrease in GLAST gene expression. However, CrMn group had a 3 fold increase in GLAST gene expression. While no direct

mechanism for this increase is known, we speculate that creatine treatment prior to Mn exposure may have provided sufficient energy reserves for particular signaling pathways that may have caused GLAST gene expression upregulation as a response to increased Mn levels in the cell. Moreover, creatine may have exerted an antioxidant role as has been suggested by recent studies, particularly in protecting nuclear DNA against oxidative damage, which thereby can affect gene regulation (Sestili et al., 2006; Guidi et al., 2008; Fimognari et al., 2009).

Since the induction of oxidative stress is a suggested mechanism for Mn neurotoxicity (Erikson et al., 2004; Milatovic et al., 2009), it is plausible that creatine could be providing cellular protection by preventing ROS generation. While excitotoxicity that results from the extracellular buildup of glutamate contributes to oxidative stress (Choi, 1988; Chen and Liao 2002), the accumulation of Mn in the mitochondria also plays part in cellular oxidative damage. The mitochondria are considered the main generator of ROS in the cell (Chance et al., 1979), and it is equipped with antioxidants that act to scavenge ROS. Glutathione is a ubiquitous tripeptide thiol that is known to participate in cellular detoxification against endogenous and exogenous oxidative stressors as well as other toxic molecules (Dringen et al., 2000). The reduced form of GSH reacts with ROS in the presence of GPx, to yield an oxidized glutathione disulfide (GSSH) and a reduced - less reactive ï oxygen species. Changes in GSH have been reported in neurodegenerative disorders and have been associated with increased oxidative stress (Gegg et al., 2003). In Mn neurotoxicity, GSH has been reported to be decreased in the striatum of juvenile rats (Erikson 2005; 2006), and in elderly rats

(Desole et al., 1997; Erikson et al., 2004). Additionally, a decrease in GSH levels has been observed in human brain glioblastoma (Park and Park, 2010) and in rat brain microvessel endothelial cell lines (dos Santos et al., 2008) after being treated with 800 εM of $MnCl_2$. The decrease in total GSH could be a marker of increased activity due to a response to oxidative stress. This decrease can also be attributed to an increase in GPx synthesis or\and activity. Using gene expression as a marker of increased GPx synthesis, previous studies in our lab, have yielded no change in GPx gene expression accompanied with no change in total GSH levels in rats exposed to 1 mg/ml $MnCl_2$ for 6 weeks via water (data not published). However, a study conducted on astrocytes by Chen and Liao (2002), reported a significant decrease in GPx gene expression in astrocytes when exposed to 500 εM of $MnCl_2$ for 24 hours. In our experiment, we yielded similar results where GPx gene expression was decreased in all Mn exposed astrocytes, and this decrease was significant in the groups that received both, Mn and creatine ($p<0.05$), where creatine does not seem to exert any protective effect. It can be assumed that the decreased gene expression of GPx could be either a result of change in cellular signaling pathways or due to disruption of the GSH recycling. Depletion of GSH has been reported in the literature to modulate gene expression (Hammond et al., 2001; Kiyosawa et al., 2007), therefore it can be speculated that the low levels of GSH may modify cellular signaling. Additionally, Mn has also been reported to cause changes in gene expression. A study using human primary astrocytes exposed to Mn, has documented a 60% decrease in glutathione reductase, the enzyme which is responsible for reducing GSSH back into GSH (Sengupta et al., 2007). Therefore, decreased glutathione reductase expression

and/or activity may lead to subsequent accumulation of GSSH that perhaps may give a negative feedback on GPx activity and gene expression. While creatine is thought to act as an antioxidant, a recent study by Juravleva et al. (2005) has demonstrated an effect of creatine on NFϖB signaling, leading to increased ROS generation rather than causing them to decrease.

Creatine was first thought to exert its protective effects through maintaining a balanced ATP/ADP ratio; however recent evidence has shown that the role of creatine in neurological tissue extends beyond sustaining energy reserves. Creatine was found to inhibit the formation of mitochondrial permeability transition pore (PTP) (Dolder et al., 2003), decrease generation of H_2O_2 by accelerating state 3 mitochondrial respiration (Meyer et al., 2006), and induce signaling pathways that enhance cell survival mechanisms (Juravleva et al., 2005). In Mn neurotoxicity, the accumulation of Mn in the mitochondria inhibits the efflux of calcium leading to an increased chance of PTP formation. Additionally, Mn was found to inhibit state 3 mitochondrial respiration by interfering with the function of Complex I of the electron transport chain (Galvani et al., 1995). Furthermore, endogenous creatine has been reported to decrease upon Mn exposure in liver tissue of rats (Fordahl et al., 2011) and in brain cell cultures (Zwingmann et al., 2003). Therefore, it can be assumed that creatine treatment may protect the cells by 1) slowing down the formation of PTP. 2) Maintaining high ATP reserves that are needed for cellular survival and defense against Mn toxicity. 3) Reducing ROS generation in the mitochondria by increasing oxygen consumption. 4) Supplying an external source of creatine to replace depleted cellular stores. These

protective mechanisms of creatine are thought to require a functional CK. A study by Watts (1963) has previously reported a loss of CK activity upon addition of Mn to the purified enzyme, where Mn replaces the enzymeôs cofactor magnesium. Meanwhile, a study on MPTP Parkinsonôs disease model deficient in mitochondrial CK has shown a neuroprotective effect of creatine, which may indicate that creatine neuroprotection may be independent from this enzyme. The effect of Mn on inhibiting both mitochondrial and cytosolic CK yet requires further investigation in an *in vitro* and *in vivo* model.

In conclusion, our data indicate that supplementing astrocytes with creatine prior to exposure to toxic levels of Mn may prevent or delay the onset of neurotoxicity by enhancing the survival of astrocytes and providing a substrate for the storage of adequate amounts of ATP that is needed for optimal cellular functioning, especially for defense against potential insults. While the use of creatine in Mn neurotoxicity is novel, studies are needed to determine dose-effect relationships in different CNS cells. Additionally, the mechanisms of the neuroprotective effects of creatine require further attention and investigation.

CHAPTER IV

EVALUTION OF THE USE OF CREATINE AS A NEUROPROTECTIVE AGENT AGAINST WATERBORNE MANGANESE-INDUCED NEUROTOXICITY IN THE RAT BRAIN

Abstract

Exposure to toxic levels of manganese (Mn) and its subsequent accumulation in the basal ganglia of the brain results in an extrapyramidal parkinsonian syndrome known as manganism. While Mn causes disrupted neurotransmission, oxidative injury and energy deficiency in the brain, and while there are no current treatments to reverse or alleviate the effect of Mn in vulnerable populations, there is constant need for research to find therapy for Mn-induced neurotoxicity. In the recent years, the use of creatine for treating neurodegenerative diseases surfaced with promising results. In this study, globus pallidus (GP), caudate-putamen (CP), substania nigra (SN) and cortex (CX) brain regions of male Sprague-Dawley rats exposed to 1 g $MnCl_2$ /L in water (Mn; n=6), given intraperitoneal injections of 75 g/kg body weight monohydrate creatine (Cr; n=6), or exposed to Mn and at the same time received creatine injections (MnCr; n=6), or exposed to deionized water with no creatine injections (Cn; n=6) were dissected and processed. The effect of waterborne exposure to Mn and creatine treatment on gene expression profiles showed no changes on GLAST mRNA levels with a statistical trend for an

increase in GLT-1 gene expression in MnCr group GP (p=0.066) and CP (p=0.052) when compared to controls, while protein levels of both GLAST and GLT-1 were not affected. A statistical trend for increased glutathione peroxidase gene expression was observed in Cr group of SN (p= 0.055) and MnCr group of CX (p=0.051), while there were no changes in total glutathione levels. Catalase gene expression was unaffected by Mn-exposure and Cr treatment; however, heme oxygenase-1 (HMX-1) in the Mn group of SN showed a statistical trend for increased gene expression (p= 0.072) when compared to controls, and GP showed a statistical trend towards an increase HMX-1 expression in Mn group (p = 0.08) when compared to MnCr group. Upon examination of creatine related genes, it was revealed that both Cr and Mn exposures did not alter the gene expression of creatine transporter and mitochondrial creatine kinase, however, brain cytosolic creatine kinase was significantly lowered in the MnCr group of the SN when compared to controls. Finally, the activity of plasma creatine kinase was unchanged in all treatment groups compared to controls. Our study suggests that, subchronic waterborne exposure to Mn does not cause significant changes on markers of oxidative stress and creatine treatment exerts some neuroprotection.

Introduction

Manganese (Mn) is a transient metal that is needed in trace amounts for sustaining activity and function of various key enzymes in the body, such as superoxide dismutase (SOD) and glutamine synthetase (GS). Manganese can be easily obtained from a diet rich in whole grains, legumes, and seeds. There is minimal evidence of Mn deficiency in humans; on the other hand, Mn toxicity leads to the development of a Parkinsonôsï like syndrome, known as manganism. This neurodegenerative syndrome characterized with bradykinesia, dystonia, rigidity, and muscular tremors, occurs due to the accumulation of Mn in the basal ganglia, which constitutes of a number of nuclei that are major regulators of movement. Specifically, Mn tends to accumulate mostly in the globus pallidus (GP), followed by the caudate-putamen (CP) (Yamada et al., 1986; Yu et al., 2003). Excessive exposure to Mn could be either airborne, where inhalation of Mn-containing fumes increases the risk of Mn accumulation in the brain; as seen mainly in welders and alloy factory workers, or exposure to Mn could be waterborne, where long term consumption of water containing 1.8-14 mg Mn/L has been associated with increased prevalence of neurological symptoms in adults, declined intellect in children, and increased infant mortality (Kondakis et al., 1986; Hafeman et al., 2007; Bouchard et al., 201l),where this is especially seen in children living in underdeveloped countries. Furthermore, the risk increases in patients suffering from liver dysfunction or suffering from anemia.

Upon its accumulation in the brain, Mn induces multiple alterations that subsequently lead to disturbed brain neurochemistry and to a putative loss of neuronal cells. One of the major changes that occur in the brain during Mn neurotoxicity is

modulation of neurotransmission in the brain, where Mn disrupts the metabolism and the uptake of glutamate (GLU), ɔ-aminobutyric acid (GABA), and dopamine. Glutamate is the major excitatory neurotransmitter in the brain; it is also a precursor for the synthesis of GABA. Extracellular levels of GLU must be maintained at low concentrations to prevent excitotoxicity (Danbolt, 2001). This clearance of GLU is carried out by astrocytes, where they metabolize GLU into glutamine through GS, which is an enzyme that requires Mn as a cofactor and that is responsible for 80% of Mn in the brain. Once glutamine is released from the astrocytes, it is taken up by neurons for the synthesis of either GLU or GABA depending on the type of neurons. The uptake of GLU into astrocytes is facilitated by active Na^+-dependent transporters, most predominantly glutamate-aspartate transporter (GLAST), and glutamate transporter-1 (GLT-1). Gene and protein expressions of these transporters are disrupted in Mn neurotoxicity leading to decreased astrocytic uptake of GLU and subsequent excitotoxicity. A study by Erikson et al. (2002) showed a decrease in astrocytic mRNA levels of GLAST upon exposure to Mn, and other studies found decreased protein levels of GLAST as well as GLT-1 in monkeys intoxicated with Mn (Erikson et al., 2007; 2008).

Besides altered neurotransmission and excitotoxicity, Mn causes an increased reactive oxygen species (ROS) generation. The tendency of Mn to accumulate in the mitochondria affects the mitochondrial physiology (Gavin et al., 1990), and disrupts energy metabolism (Zwingmann et al., 2003). It has been reported previously that Mn causes mitochondrial dysfunction by disrupting calcium homeostasis and by inhibiting enzymes involved in the electron transport chain as well as in the Krebôs cycle, where

these changes contribute to increased oxidative stress, energy failure, and to the induction of apoptosis. Along with the free radicals that are generated from the mitochondria, Mn is thought to possess a pro-oxidant activity, where it causes auto-oxidation of dopamine (Florence and Stauber, 1989), and peroxidation of lipids (Avila et al., 2008). Furthermore, protective antioxidant systems in CNS cells are targeted by Mn neurotoxicity. For instance, levels of glutathione (GSH), a thiol antioxidant that scavenges free radicals in the presence of glutathione peroxidase (GPx), were reported to be changed in the CP, and cortex (CX) of monkeys exposed to Mn via air, where these changes were dependent on duration of exposure (Erikson et al., 2007; 2008). Additionally, other endogenous antioxidant systems such as metallothionein and SOD have been reported to be altered too (Erikson et al., 2006).

Heme Oxygenase-1 (HMX-1) is an endoplasmic reticulum enzyme that is upregulated under oxidative stress conditions, where its correspondent gene is known to contain stress response elements and metal response elements, therefore rendering it sensitive for induction by pro-oxidants and by heavy metals (Dennery et al., 2000). Upon increased oxidative stress, there is an increase in ñfree heme poolsò in the cell, where it is thought that the upregulation of HMX-1during stress is intended to protect against heme pro-oxidant activity by breaking it down into biliverdin, which further breaks down into bilirubin, where these two compounds are considered intracellular antioxidants (Ryter and Tyrrell, 2000). While the gene expression of this enzyme has been investigated in other neruodegenerative diseases, it is unknown whether it is upregulated during Mn neurotoxicity and whether it influences the disrupted metabolism of iron that has been

reported in previous studies. Catalase is an iron-dependent enzyme that acts as an antioxidant by converting H_2O_2 into H_2O and O_2 (Michiels et al., 1994). The activity of catalase has been previously reported to be altered (Liccione and Maines, 1988); therefore it is important to further investigate the effect of Mn-exposure on the gene expression of this antioxidant.

Creatine is a guanidino compound that participates in cellular energy balance via the creatine-phosphocreatine shuttle. Whether synthesized intracellularly or taken up from the blood via creatine transporter (CRT-1), creatine obtains a phosphate group from ATP in a reaction catalyzed by creatine kinases (CKs), leading to the formation of phosphocreatine and ADP. Whenever a sudden increase for energy arises, CKs mobilize the reaction in the opposite direction generating ATP for energy demanding sites in the cell and releasing creatine. Interestingly, in the recent years this ergonomic compound, which is used mainly as a supplement for athletes, has been introduced as a potential neuroprotective agent against neurodegenerative disorders in lab as well as in clinical trials (Klivenyi et al., 1999; Matthews et al., 1999; Bender et al., 2005; 2006). It has been suggested that besides its contribution to cellular energy equilibrium, creatine has a potential to protect against mitochondria-induced apoptosis (Dodler et al., 2003). Furthermore, studies have proposed antioxidant activity of creatine (Fimognari et al. 2009), and a possible involvement in key signaling pathways, such as NFϽB pathway (Juravleva et al., 2005).

While there are no current treatments for Mn neurotoxicity, the successful emerging use of creatine in Parkinsonôs disease that has a similar pathophysiology to

manganism, encourages the idea of investigating the use of creatine for treating Mn neurotoxicity. Therefore, the aims of this study are (1) to examine the use of creatine as a neuroprotective agent in an in vivo model of waterborne Mn exposure; (2) to investigate the effects of waterborne Mn exposure and creatine treatment on brain gene expression profiles of GLU transporters (GLAST and GLT-1), oxidative stress enzymes (GPx, catalase, and HMX-1), and on CKs as well as CRT-1; and (3) to identify brain changes in GLU transporters protein expression (GLAST and GLT-1), total GSH levels, and plasma CK activity.

Materials and Methods

Animals

Male weanling (21 days old) Sprague-Dawley rats (Harlen Sprague-Dawley, Indianapolis, IN) (*n= 24*) were randomly divided into four groups: controls (Cn; AIN-93G diet and deionized water), Mn-exposed controls (Mn; AIN-93G diet and deionized water containing 1 g $MnCl_2$/ L), creatine-treated controls (Cr; AIN-93G diet and deionized water), and Mn-exposed creatine-treated group (MnCr; AIN-93G diet and deionized water containing 1 g $MnCl_2$/ L). Diets were obtained from Dyets Inc. (Bethlehem, PA), and certified for metal content. The Mn-exposure protocol used has been previously proven in our lab to achieve brain Mn accumulation (Anderson et al., 2007; 2008; Fordahl et al., 2010). Rats had free access to food and water 24 hour/day, and were housed in hanging cages in an animal facility with a typical dark light cycle (1800 hour - 0600 hour lights off) and a room temperature maintained at 25± 1 °C. Animals were acclimated for 1 week prior to the experiment. Creatine monohydrate

(Thermo Fisher Scientific, NJ) was dissolved in saline (30 mg/ml), and 75 mg/kg body weight was injected intraperitoneally in creatine-treated rats (Cr and MnCr groups), whereas other groups received only saline. At the end of the experiment (6 weeks), the rats were sacrificed, and brain tissue was dissected into the following regions: globus pallidus (GP), caudate-putamen (CP), substania nigra (SN) and cerebral cortex (CX). Tissues were quick frozen on dry ice, and stored at -80 °C for later use. All animal protocols were approved by the University of North Carolina at Greensboro Animal Care and Use Committee.

Metal Analysis

Concentrations of Mn and iron (Fe) were measured using graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). Brain tissues were digested in ultrapure nitric acid (1:10, wt/vol. dilution) for 48-72 hours in a sand bath at 60° C. An additional dilution (1:20) with 2% nitric acid was made prior to the analysis. Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 ε g Mn/g; 184 ε g Fe/g) was digested in ultrapure nitric acid and used as an internal standard for analysis (final concentration 5 ε g Mn/L; 92 ε g Fe/L).

RNA Isolation and cDNA Synthesis

Tissues intended to be used for gene expression assays were submerged in RNA*later*® solution (1:10 wt/vol dilution, Ambion Inc., Austin, TX), and stored at -80 C° until analysis. After samples were homogenized, RNA was isolated using ToTALLY RNAÊ kit (Ambion Inc., Austin, TX) following the manufacturerôs instructions. Prior to cDNA synthesis, RNA concentration and purity were determined using A260:A280 ratio

produced by NanoDrop spectrophotometry (Thermo Scientific, Delaware, MD). For cDNA synthesis, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used, where 1 εg of total RNA was incubated with MultiScribeÊ Reverse Transcriptase (50 U), 10× Random Primers, 25×dNTP Mix (100 mM), and RNase Inhibitor at room temperature for 10 minutes followed by 1 hour at 37° C in a final volume of 20 εl.

RT-PCR

TaqMan® Gene Expression Assays were purchased to probe for GLAST, GLT-1, CRT-1, CK-BB, CK-Mit1, HMX-1, GPX, and catalase gene expression, in conjunction with Universal PCR Master Mix (Applied Biosystems, Foster City CA). Triplicate aliquots of cDNA were analyzed on 96-well plates using the expression assays for the genes of interest. All gene expression values were normalized to ϐ-actin expression value from the same sample on the same plate and reported as percent of control.

Protein Extraction

Tissue samples were sonicated in 500 µl of RIPA lysis buffer (1% Nonidet 40, 1% SDS, 0.5% sodium deoxycholate, 1 mM NaF, 2 mM ß-glycerolphosphate, 1 mM sodium orthovanadate, and 1X protease inhibitor cocktail (Chemicon, Houston, TX) in 1X PBS) on ice until completely homogenized. Homogenates were incubated on ice for 20 minutes before being centrifuged at 12,500 x g for 20 minutes at 4ęC. Supernatant was then transferred to new tubes and used to determine total protein concentration by BCA assay (Pierce, Rockford, IL) before proceeding with western blot analysis.

Western Blot Analysis

Western blot analysis was conducted to examine the effects of manganese and creatine on the expression of GLAST and GLT-1 proteins *in vivo*. Protein samples (20 µg) from GP and CP were combined with 4X LDS sample buffer (Invitrogen, Carlsbad, CA) containing 5% ß-mercaptoethanol and heated at 70ęC in a heat block for 10 minutes. Samples were then loaded onto a 4-12% Bis-Tris pre-cast polyacrylamide mini gel (Invitrogen, Carlsbad, CA) and electrophoretically separated under denaturing conditions in 1X MES buffer containing 1% antioxidant (Invitrogen, Carlsbad, CA). Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) before blocking with 5% non-fat milk. Membranes were probed overnight at 4ęC with primary antibody (rabbit polyclonal anti-rat GLAST 1:1500; rabbit polyclonal anti-rat GLT-1 1:4000; rabbit polyclonal anti-ɓ-actin 1:8000, Alpha Diagnostics International, San Antonio, TX) for the protein of interest in 5% BSA. Membranes were rinsed in 1X TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 1.5% Tween 20) and probed for 2 hours at room temperature with an HRP-conjugated secondary antibody (goat anti-rabbit 1:10000, Alpha Diagnostics International, San Antonio, TX) in 5% BSA. Membranes were then rinsed several times in 1X TBST before incubation in ECL solution (Perkin Elmer, Waltham, MA) and exposure to radiographic film (Pierce, Rockford, IL). ß-actin housekeeping protein was probed on the membrane to attest equal loading of protein samples and for image analysis. Recombinant peptide controls for GLAST and GLT-1 were used to help identify target bands. Films were analyzed using image analysis software (ImageJ, NIH,

Bethesda, MD), with the amount of the target protein from each sample standardized to the amount of ß-actin from the sample.

Total GSH

Tissues were diluted 1:10 wt/vol. in phosphate buffered saline (pH 7.4). Following homogenization via sonication, the samples were centrifuged at 10,000 x g for 15 minutes. Supernatant was transferred to new tubes and deproteinized by adding an equal amount of metaphosphoric acid (5 g dissolved in 50 ml water) (Sigma-Aldrich Inc., St. Louis, MO), then samples were vortexed for 30 seconds, and centrifuged at 3,000 x g for 2 minutes. To increase the sample pH, a 50 εl of 4 M triethalolamine (TEAM) (Sigma-Aldrich Inc., St. Louis, MO) was added per ml of supernatant. Total glutathione levels were measured immediately after the addition of TEAM reagent using Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI). Results are expressed as total glutathione equivalents per mg protein.

Plasma CK Activity

Creatine kinase colorimetric activity assay kit was obtained from BioAssay Systems (Haward, CA). Trunk blood was collected and centrifuged for 10 min at 12,000 rpm speed. Plasma was then moved to new tubes and stored at -80° C. Later, the plasma samples were thawed and 10 εL of sample were mixed with 100 εL of the assay reagent. After 20 and 40 minutes incubation at 37° C, absorbance was measured at wavelength of 340 nm. Calculations using absorbance of a provided calibrator and of water were used to determine creatine kinase activity expressed as unit per liter (U/L), where one unit is

equivalent to the transfer of 1 εmole of phosphate from phosphocreatine to ADP per min at pH 6.0. The assay was repeated twice with the samples ran in triplicates.

Statistical Analysis

Statistical analysis was conducted using SPSS version 14 for windows (Chicago, IL). Data were analyzed using a two-way ANOVA to detect differences among groups, and Dunnettôs post hoc analysis was used to determine difference from controls when $p < 0.05$. Trends towards statistical significance were appointed at $p < 0.1$. Outliers were removed via boxplot analysis with ± 2 standard deviation criteria. Two-tailed Pearsonôs correlations analyses were conducted to identify relationships between Mn concentrations, protein and mRNA levels.

Results

Metal Concentrations

Levels of Mn were significantly elevated in Mn-exposed groups (Mn and MnCr) compared to controls in the GP and CP, whereas a non-significant increase was noted in SN and CX (Figure 4-1, Table 4-1). Approximately a 40% decrease in Mn concentration was observed in Cr group of GP when compared to control, however this change was not statistically significant. No changes were seen in Fe levels in the four brain regions (Figure 4-2, Table 4-2).

Table 4-1: Manganese Concentrations

	GP	CP	SN	CX
CN	0.2735503 ± 0.0364976	0.3084356 ± 0.0193622	0.300782 ± 0.0653443	0.4064485 ± 0.0731025
Mn	0.5642267 ± 0.0635143*	0.5661074 ± 0.0447178*	0.5491050 ± 0.0679561	0.6296066 ± 0.1486872
Cr	0.1664672 ± 0.0577272	0.2986443 ± 0.0358098	0.2511348 ± 0.0795295	0.4437132 ± 0.2114589
MnCr	0.4822331 ± 0.0441297*	0.6715911 ± 0.1195424*	0.4101785 ± 0.0573310	0.4456496 ± 0.048363

Mn concentrations expressed as μg /g tissue.
** Statistically significant at $p < 0.05$*

Table 4-2: Iron Concentrations

	GP	CP
CN	8.72122756 ± 1.4045252	9.1133666 ±0.9971788
Mn	8.3267558 ± 1.6895308	10.16853 ± 1.5130574
Cr	7.8352981 ± 1.2968245	11.59390 ± 1.3702259
MnCr	8.7575973 ± 1.6797409	11.216222 ± 0.8809103

Fe concentrations expressed as μg / g tissue.
** Statistically significant at $p < 0.05$*

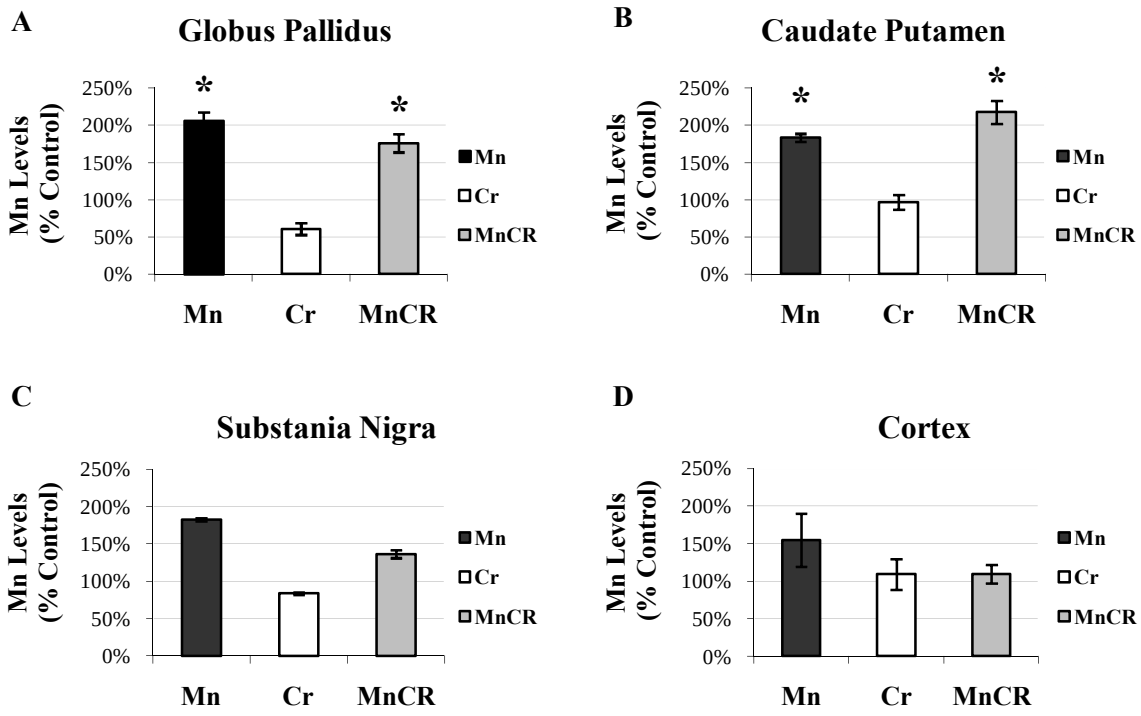


Figure 4-1: Brain Manganese Concentrations. Concentrations of Mn in the brain, where Mn values are expressed as percent control ± S.E.M. , where mean control values are equivalent to ñ100%ò. Mn exposure significantly increased Mn accumulation in the (A) globus pallidus and (B) caudate-putamen versus control. Mn levels were increased also in Mn-exposed groups in (C) substania nigra and (D) cortex when compared to controls but without statistical significance. *$p < 0.05$ according to Dunnettôs post hoc analysis.

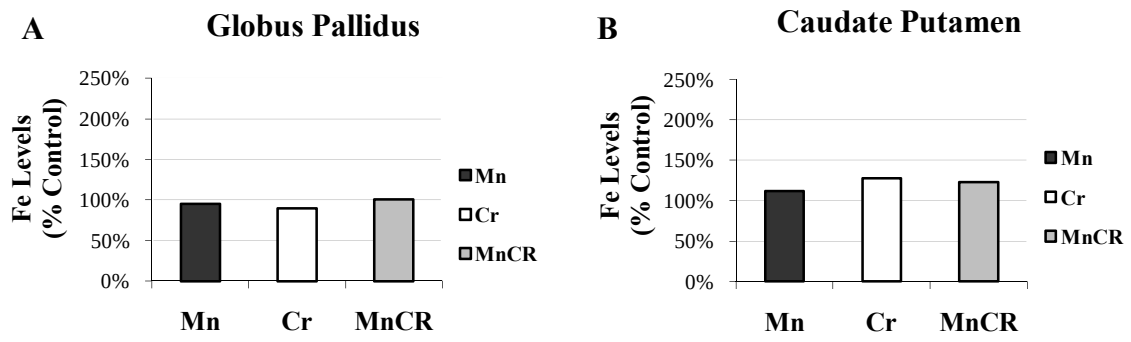


Figure 4-2: Brain Iron Concentrations. Concentrations of Fe in the brain, where Fe values are expressed as percent control ± S.E.M., where mean control values are equivalent to ñ100%ò. Fe exposure was not altered in any of the treatment groups in (A) globus pallidus and (B) caudate-putamen when compare to control. *$p < 0.05$ according to Dunnettôs post hoc analysis.

GLAST and GLT-1 Gene Expressions

GLAST gene expression did not change in any of the groups in all brain regions. On the other hand, GLT-1 gene expression yielded a statistical trend for an increase in MnCr groups of both the GP ($p = 0.066$) and CP ($p = 0.052$), when compared to controls. No changes were observed in other groups or in SN and CX (Figure 4-3).

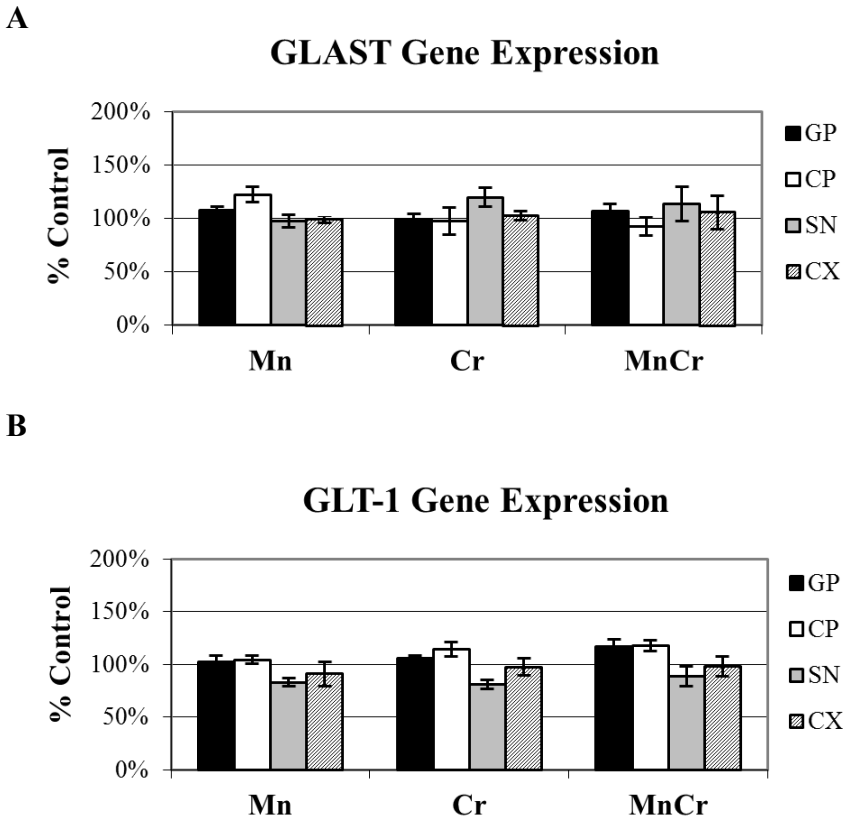


Figure 4-3: Gene Expression of GLAST and GLT-1. Gene expression of (A) GLAST and (B) GLT-1 in globus pallidus, caudate-putamen, substania nigra, and cortex, where values are expressed as percent control ± S.E.M. with mean control values equivalent to ñ100%ò. (A) No changes were found in GLAST gene expression. (B) A non-significant ~ 20% increase in GLT-1 gene expression was observed in MnCr group of GP and CP, while no other changes were observed in other groups or other brain regions.

GPx, Catalase, and HMX-1 Gene Expressions

Statistical significance trends for increased GPx gene expression were observed in Cr group of the SN (p=0.055) and in MnCr group of the CX (p =0.051), when compared to controls. No other changes were seen in GPx gene expression. No changes were found in catalase gene expression due to Mn or creatine treatments. For HMX-1 gene expression, a statistical trend towards an increase was seen in the Mn group when compared to MnCr in the GP (p=0.08). Also, in the SN, Mn group tended to have an increase in gene expression when compared to controls, however this increase was only a trend towards significance (p=0.072). No other changes were seen (Figure 4-4).

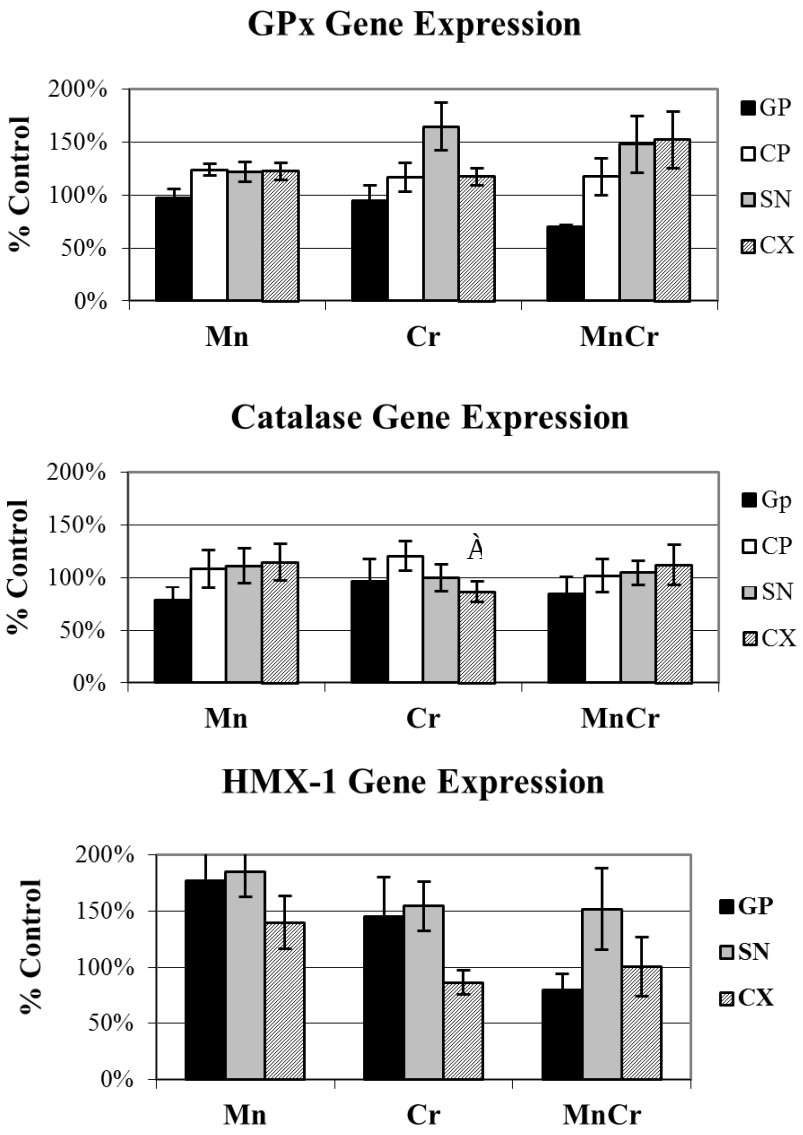


Figure 4-4: Gene Expression of GPx, catalase, and HMX-1. Gene expression of (A) GPx , (B) catalase, and (C) HMX-1 in globus pallidus, caudate-putamen, substania nigra, and cortex, where values are expressed as percent control ± S.E.M. with mean control values equivalent to ñ100%ò. (A) No changes were obsereved with GPx gene expression, except for non-significant increase seen in Cr group of Sn and MnCr group of CX, when compared to controls. (B) No changes were seen in catalase gene expression. (C) An approximate non-significant 50% increase was observed in gene expression of HMX-1 in Mn group of GP.

CRT-1, CK-BB, and CK-Mit1 Gene Expressions

Gene expression of CRT-1 and CK-Mit1 were not altered in any treatment group compared to control. However, changes in CK-BB gene expression were observed in the CP, where it was significantly lowered in MnCr when compared to controls (p=0.007) Figure (4-5).

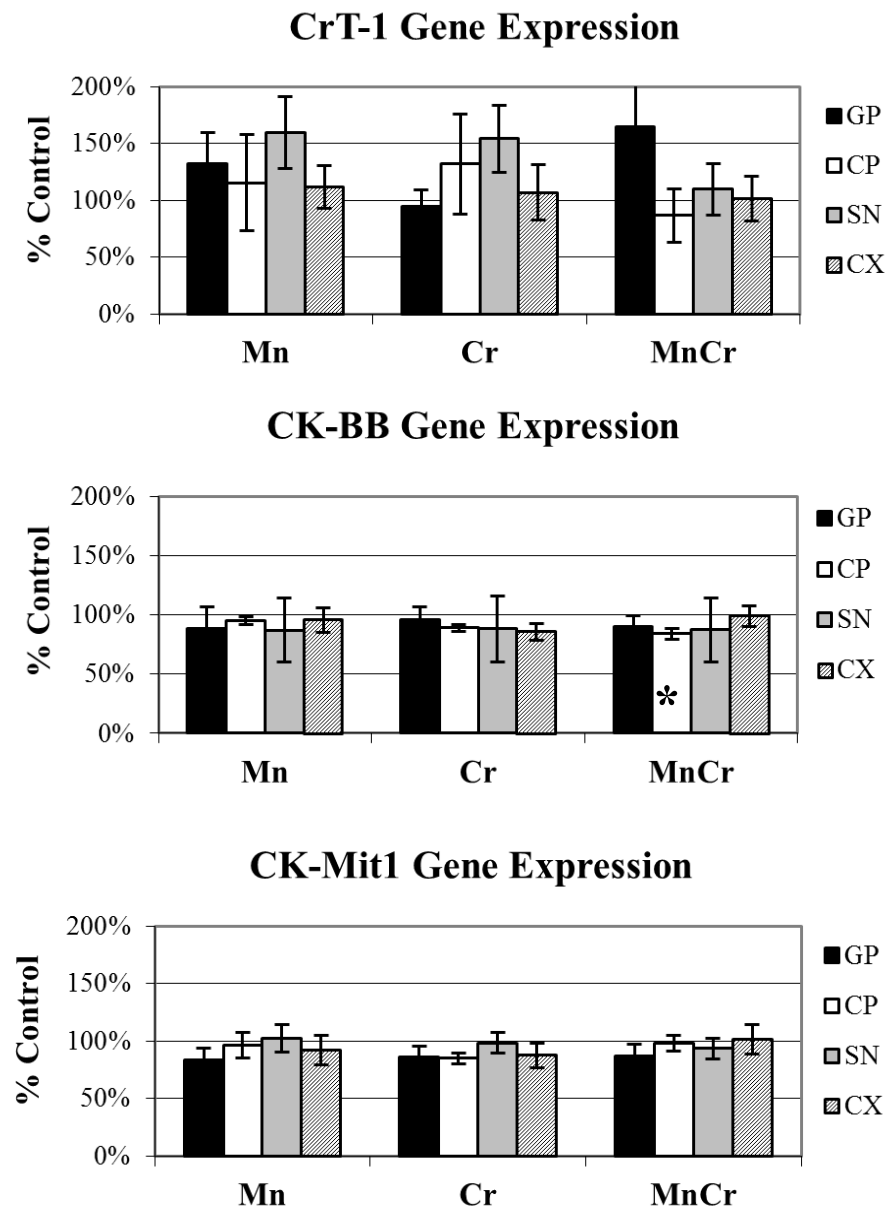
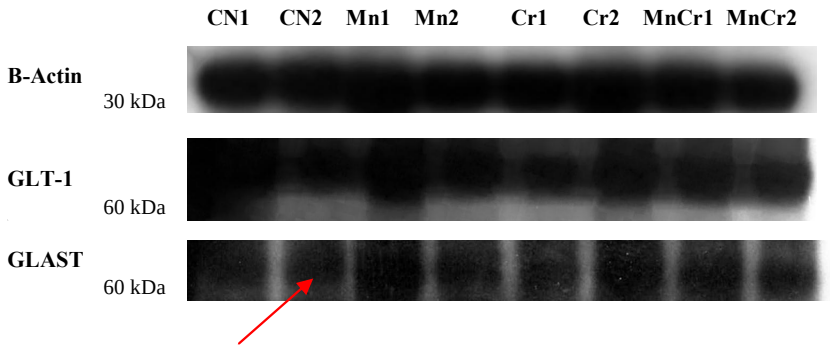


Figure 4-5: Gene Expression of CRT-1, CK-BB, and CK-Mit1. Gene expression of (A) CRT-1, (B) CK-BB, and (C) CK-Mit1 in globus pallidus, caudate-putamen, substania nigra, and cortex, where values are expressed as percent control ± S.E.M. with mean control values equivalent to ñ100%ò. (A) No changes were found in CRT-1 gene expression. (B) A significant decrease in CK-BB gene expression was observed in MnCr group of SN group ($p>0.05$), and a similar near-significant decrease was also seen in MnCr group of CP, no changes were observed in other groups. (C) No changes were seen for the gene expression of CKMit1. *$p < 0.05$ according to Dunnettôs post hoc analysis.

Western Blot Analysis

No Changes were observed in GLAST and GLT-1 proteins in GP and CP (Figure 4-6).

(A) Globus pallidus western blots



(B) Caudate-putamen western blots

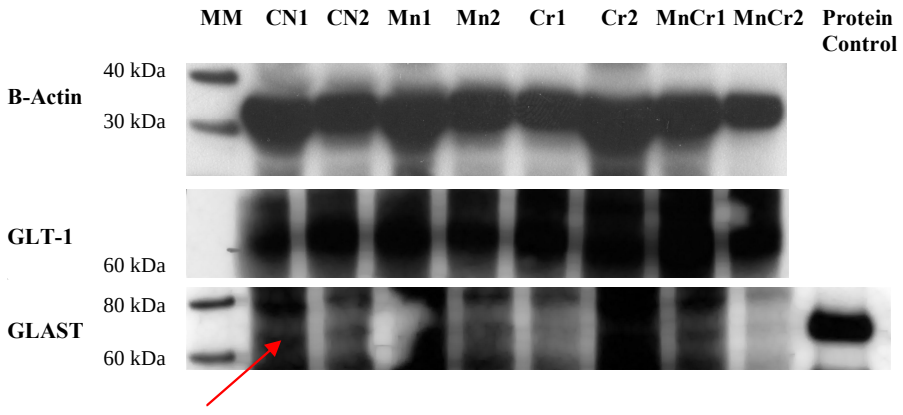


Figure 4-6: Western Blots of GLAST and GLT-1 Proteins. (A) Globus pallidus. (B) Caudate-putamen. No significant changes were seen in GLAST and GLT-1 protein concentrations.

Total GSH

Levels of glutathione remained unchanged in the CP, however, a slight non-significant decrease was observed in the GP (Figure 4-7).

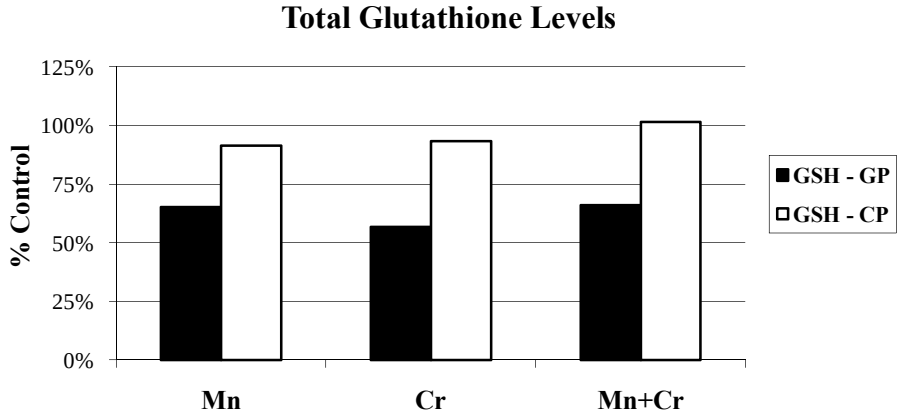


Figure 4-7: Total Glutathione Levels. Total glutathione levels in (A) globus pallidus, and (B) caudate-putamen, where values are expressed as percent control ± S.E.M. with mean control values equivalent to ñ100%ò. (A) A non-significant decrease was observed in all groups in GSH levels when compared to control in the GP. (B) No changes were seen in the CP GSH levels.

Creatine Kinase Activity

Although CK activity in the plasma increased in Cr group, this increase was not statistically significant. Furthermore, no changes were observed in Mn and MnCr groups despite the increase in plasma Mn concentrations (Figure 4-8)

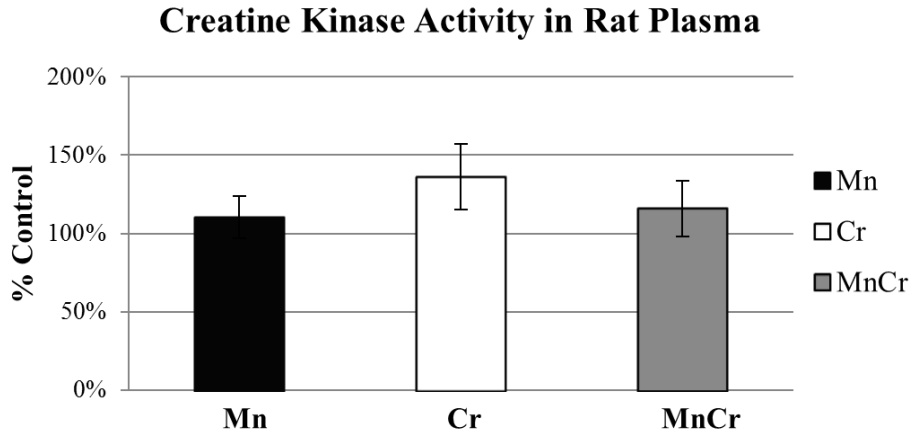


Figure 4-8: Creatine Kinase Activity in Plasma. Creatine kinase activity values expressed as percent control ± S.E.M. with mean control values equivalent to ñ100%ò. No Changes were observed in Mn and MnCr groups, where as a non-significant increase was noted in the Cr group.

Discussion

Manganese is known to induce GLU excitotoxicity through decreased GLU uptake by astrocytes (Hazell and Norenberg, 1997). Alterations in gene and protein expression of both GLAST and GLT-1 have been reported previously in Mn-exposed nonhuman primates. Erikson et al. (2007) showed a pallidal increase in GLAST and GLT-1 gene expression with decreased GLAST proteins, and a decrease in GLT-1 mRNA levels and protein in caudate. Meanwhile, in our study we report no significant changes in gene expression and protein levels of GLAST and GLT-1 in GP, CP, SN, and CX brain regions upon waterborne exposure to Mn, and our data corroborates the unaltered protein profiles found in monkeys that received weekly Mn injections (Burton et al., 2009). Furthermore, initial stages of altered GLU uptake may not necessary be due to changes in gene or protein expressions, rather it could be due to ion imbalance, especially that GLU transporters are dependent on intact Na^+, K^+, and H+ gradients (Billups et al., 1998; Choi, 1988), which may be disrupted in Mn toxicity (Scheuhammer and Cherian, 1981). It can be hypothesized that Mn may induce a post-translational modification of GLAST and GLT-1 proteins, or even it may bind non-competitively to these proteins therefore affecting their activity rather than concentrations (Hazell and Norenberg, 1997).

Given its fatty composition, the brain is known to be highly vulnerable to lipid peroxidation, which is a marker of neurodegenerative diseases. Besides increased oxidative stressors in Mn neurotoxicity, antioxidant defense mechanisms have been repeatedly reported to be altered (Dobson et al., 2003; Taylor et al., 2006). Glutathione,

an antioxidant synthesized from L-cysteine, L-glutamic acid, and glycine, can scavenge free radicals to rid the cells of their potency to cause oxidative damage, where GPx is required for this process to occur. The GSH system in models of Mn neurotoxicity is altered, where inhalation studies of nonhuman primates have previously reported a decrease in GSH levels in the caudate post exposure to Mn, particularly in animals of older age (Erikson et al., 2007). Meanwhile in a different study, a sub-chronic oral exposure to Mn was found to decrease GSH levels in CP of aged rats, but increase it in younger ones (Desole et al., 1995). In our study we report that waterborne exposure to Mn causes no changes in total levels of GSH in the CP with a non-significant approximate 30% decrease in the GP. Findings in the CP corroborate a study by Dobson et al. (2003), where airborne exposure to Mn did not induce any changes in GSH levels of the CP in rats. However, a study published in 2008 showing a decrease in GSH levels in the caudate and an increase in the putamen of monkeys exposed to Mn, arouses the possibility that opposite changes in the caudate and the putamen may neutralize the results (Erikson et al., 2008). Furthermore, we assessed GPx gene expression and found no significant changes upon exposure to Mn corroborating a similar study conducted previously in our lab (unpublished data). The activity of catalase, an antioxidant, is reported to decrease in Mn neurotoxicity (Liccione and Maines, 1988); therefore it can be assumed that the gene expression of this enzyme will elevate upon increased ROS in the brain, we measured it in the four brain region, but found no changes affiliated with exposure to Mn.

Overexpression of HMX-1 is associated with increased oxidative stress, and has been documented in a number of neurodegenerative diseases such as Parkinsonôs disease and Huntingtonôs disease (Schipper, 2004). Recently, Li and colleagues (2011) reported increased HMX-1 proteins in PC12 cells exposed to Mn that are possibly related to the activation of the NF-E2 factor 2 (Nrf2) signaling pathway by Mn. In our study we report a 50% non-significant increase in HMX-1 gene expression in GP (p = 0.240), and SN (p=0.072). Although these findings are not statistically significant, the overexpression of HMX-1, especially in the SN may indicate a possible role of HMX-1 in the progression of Mn-induced neurotoxicity, where there is an ongoing debate of whether the overexpression of HMX-1 is neuroprotective or is detrimental to cells by increasing labile Fe.

Creatine is a nitrogenous organic acid that is important for energy maintenance in tissues with high fluctuating energy demands such as the brain, which depends on both endogenously synthesized creatine, and creatine obtained from the blood circulation via the blood brain barrier. Previous reports have shown a decrease in creatine content of the liver as well as the brain upon intoxication with Mn (Dorman et al., 2008; Fordahl et al., 2011); therefore supplying an exogenous source of creatine may prevent such deficiency and enhance the energy status of the cell. In our study, treating Mn-exposed rats with creatine showed a slight enhancement of gene expressions of GLT-1 in GP and CP, GPx in CP, and HMX-1 in SN where these changes signify a neuroprotective role of creatine in reversing changes that are induced by Mn in the brain. While these changes were

mostly statistical trends, it is possible that the small sample size and the short duration of exposure to Mn have influenced our findings.

Multiple mechanisms have been proposed for the therapeutic implications of creatine in neurodegeneration. It is thought that the contribution of creatine in generating ADP to provide a positive feedback on the electron transport chain decreases the release of ROS from the mitochondria, where this process is coupled to the mitochondrial CK (Meyer et al., 2006). Moreover, it has been suggested that the role of creatine in energy homeostasis not only decreases ROS generation and increases ATP supplies, but also imposes protection against the development of the mitochondrial permeability transition pore (PTP), which is one of the late events occurring in Mn cytotoxicity. This modest protection against PTP is also thought to be attributed to mitochondrial CK which is an octamor that is bound to the mitochondrial inner and outer membranes and is thought to be coupled with antinuclear translocase (Dodler et al., 2003; Speer et al., 2005). However, creatine administered to mitochondrial CK-deficient mice yielded significant neuroprotection against 1-methyl-1, 2, 3, 6-tetrahydropyridine toxicity indicating that the protective effects of creatine may be attributed to other mechanism or to the cytosolic CK rather than the mitochondrial (Klivenyi et al., 2004). In our study, we measured gene expression of both the cytosolic and the mitochondrial isoforms of CK as well as CRT-1, however no changes were found except for a significant decrease in CK-BB in MnCr group of the SN. Moreover, measurements of plasma creatine kinase activity only yielded a slight increase in activity in MnCr group with an approximate 30% increase in Cr group, where it was expected that Mn would decrease the activity of CK given its

cysteine-containing structure that makes it vulnerable to oxidants, as well as a the possible competitive binding of Mn to Mg-binding site on CK, where it is well documented that Mn interferes with the binding of Mg in multiple enzymes and receptors such as N-methyl-D-aspartate receptor (Chan et al., 1992)

In conclusion, waterborne sub-chronic exposure to Mn in rats may not impose major neurochemical alterations related to GLU neurotransmitter transporters as well as to oxidative stress defense systemsôgene expression and protein concentrations, however it is obvious that this exposure initiates minor neurotoxic events within the CNS that involve HMX-1 enzyme. Furthermore, creatine treatment seems to have some sort of neuroprotection against Mn-induced neurotoxicity, yet there is a need to investigate the use of higher dosage in different models of Mn-exposure and perhaps with the use of intracranial or oral administration of creatine.

CHAPTER V

EPILOGUE

Despite the essentiality of manganese (Mn) to biological systems, excess Mn is detrimental to normal neurological health of human beings. Manganese-induced neurotoxicity is marked with pallidal cell loss and degeneration that follows disruption of the basal ganglia inhibitory and excitatory neurotransmission pathways, where Mn interferes with neurotransmitter transport system as well as enzymatic activity of neurotransmitter-metabolizing enzymes, such as glutamine synthetase (Erikson and Aschner, 2003; Fitsanakis et al., 2006). Another deleterious feature of Mn neurotoxicity is increased generation of reactive oxygen species and the subsequent oxidative damage that hinders astrocytic and neuronal functioning (Taylor et al., 2006; Milatovic et al., 2009). These changes, along with Mn-induced mineral and ion imbalance, contribute to energy failure and to induction of apoptosis, thereafter causing neurodegeneration (Scheuhammer and Cherian, 1981; Zwingmann et al., 2003).

The purpose of this study was to further characterize the effect of Mn accumulation on glutamate (GLU) transport system, and on endogenous antioxidant systems with and without the administration of the energy substrate creatine. We examined the effect of pre- and post creatine treatment on survival of Mn-exposed astrocytes, and measured mRNA levels of glutamate-aspartate transporter (GLAST), glutathione peroxidase (GPx), and creatine transporter-1 (CRT-1). We also assessed

creatine therapy during waterborne Mn exposure in rats, and measured end points to assess changes in GLU transporters, oxidative stress markers, and creatine function.

We found that creatine enhanced cellular survival especially when administered prior to Mn exposure in astrocytes despite elevated intracellular Mn levels. Meanwhile, the marked increase that we observed in GLAST gene expression must be interpreted with caution. This is because GLAST expression in tissue could be influenced by neuronal-glial interactions, where the regulation of GLU transporters gene expression is influenced by many factors that include GLU extra- and intracellular concentrations (Billups et al., 1998; Ye and Sontheimer, 2002). Moreover, our results showed that astrocytes exposed to both creatine and Mn had a decrease in GPx gene expression, which brings about a question regarding the oxidative stress status of the cells when treated with Mn and creatine. Interestingly, the available literature on creatine contradicts itself, where some researchers suggest antioxidant activity of creatine (Fimognari et al. 2009), while others propose its contribution to increased ROS generation (Juravleva et al., 2005). Therefore, a direct measure of free radical concentrations would further clarify the protective effects of creatine.

Oral exposure to Mn did not induce significant changes in GLAST and GLT-1 protein regardless of the significant elevation in Mn concentrations, namely in the globus pallidus and the caudate-putamen. This lack of change could be due to a short duration of exposure to Mn (42 days) which might not have allowed enough time for protein turnover to occur and therefore to demonstrate changes. Furthermore, past studies have demonstrated that the different forms of Mn and different routes of exposure may have

different absorption trends and accumulation patterns in the CNS, and therefore perhaps may have different neurotoxic behaviors (Roels et al., 1997). However, for more accurate measurements of the influence of Mn on GLU transporters, it is preferable to measure protein activity levels instead of total protein content. Changes in some oxidative stress markers were also minimal following waterborne Mn exposure mainly in the GSH and catalase antioxidant systems. Alteration in total GSH is documented to be age- and sex-dependent, where older animals tend to have an overall decline in the brainôs basal GSH concentrations (Maher, 2005). Moreover, it is important to point out that our measurements of total GSH did not designate levels of oxidized versus reduced GSH, which may not necessary reflect true status of GSH in the tissue. Meanwhile, our novel finding of a near significant Mn induction of gene expression of HMX-1 corroborated previous data on the role of Mn in activating NF-E2 factor 2 (Nrf2) signaling pathway that up-regulates HMX-1 expression (Li et al., 2011), bringing forth more questions about the role of HMX-1 in oxidative stress and altered iron metabolism in Mn neurotoxicity.

While the administration of creatine treatment showed effectiveness in astrocyte cell cultures, it caused no or minimal non-significant changes in the Mn-exposed rat brain. This could be attributed to the low dosage of creatine used; especially that creatine monohydrate is characterized with low solubility in aqueous solutions that renders the application of high dosage if administered via intraperitoneal injections. Additionally, a previous study using the maximum soluble creatine concentrations revealed that intraperitoneal injections only increase the brainôs concentrations by 70 ε M, which is not

high enough to have a therapeutic impact on the brain (Perasso et al., 2003). Meanwhile, it is possible that intracerebroventricular administration of creatine may increase the brainôs creatine levels, where this increase has been found comparable to creatine concentrations needed for protection against ischemia (Rebaudo et al., 2000). The use of a different creatine-derivative may also be another alternative for examining the protective role of creatine in vivo models of Mn neurotoxicity. For instance, phosphocreatine-Mg complex acetate is a creatine derivative that has been found to cross the blood brain barrier and protect against ischemia in mice (Perasso et al., 2009).

Our findings suggest that creatine has some neuroprotection against Mn-induced neurotoxicity, similar to that seen in other neurodegenerative diseases. However, the mechanisms of action for neuroprotection remain unclear and demand further investigation. Despite the effect of creatine on cell survival on Mn-exposed astrocytes, it is important to measure the uptake of creatine to determine the dosage required to obtain neuroprotection. This may help with determining an effective dose to be used in animals. Furthermore, with existing evidence of neuroprotective role of creatine against excitotoxicity in other neurodegenerative diseases (Bender et al., 2005; Andreassen 2001b), it is important to determine GLU uptake and concentrations in models of Mn neurotoxicity that are treated with creatine. Because creatine was found to protect N-methyl-D-aspartic acid (NMDA) receptor against neurodegeneratione (Malcon et al., 2000), and since this receptor alteration in Mn neurotoxicity contributes to induction of excitotoxicity and ion imbalance, it is worthwhile to assess the influence of creatine on NMDA in Mn-induced neurodegeneration.

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